University of Lisbon

Faculty of Pharmacy

In vitro infection of lymphoid tissues by HIV-2

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The dissertation was supervised by
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Abstract

Lymphoid organs constitute an optimal environment for the defense against the invasion of pathogens by promoting immune responses. They are the major reservoirs of human immunodeficiency virus (HIV) infection, however their infection has only been focused in the context of HIV-1. HIV-2 represents a more attenuated form of HIV disease, characterized by slower $CD4^+$ T cell decline and progression to AIDS, that doesn't have the same impact worldwide as HIV-1. Nevertheless, HIV-2 constitutes a unique naturally occurring model of attenuated HIV disease valuable for the study of HIV pathogenesis. Understanding the impact of HIV-2 infection on lymphoid organs will provide new insights regarding the ability of the tissue to promote immune responses.

Here we investigated the impact of HIV-2 in human tonsillar tissue, a secondary lymphoid organ (SLO). Tonsil organ cultures (TOCs) were infected with HIV-2 or HIV-1 primary isolates using either CCR5 (R5) or CXCR4 (X4) coreceptors. All viruses were able to replicate in the tissue, as revealed by immunohistochemistry. In addition, we found that HIV-2-infected lymphocytes from TOCs presented similar levels of total HIV DNA as HIV-1. Surprisingly, Gag mRNA and protein levels were significantly higher in tissue infection by HIV-1 X4 as compared to R5-tropic HIV-1 or HIV-2, suggesting that the viral production observed in TOCs may be due to the infection of other cells in the tissue, and that viral transcriptional regulation may be altered in X4-tropic HIV-2. Interestingly, despite the lower lymphocyte-associated viral replication observed with X4-tropic HIV-2, the impact on the viability of $CD4^+$ T cells and on the memory $CD4^+$ T cell compartment was similar to that observed with HIV-1 X4. Moreover, we found that infection of TOCs with X4-tropic viruses resulted in a higher frequency of cells expressing Foxp3, a molecule related with the regulatory phenotype. This was particularly observed for X4-tropic HIV-2 infection and was not related with cell proliferation. In addition, we observed a significantly higher depletion of follicular cells within $Foxp3^+$ cells in TOCs infected with X4-tropic HIV-2, indicating higher persistence of $Foxp3^+$ Tregs. Finally, we observed that all viruses were able to deplete the T follicular helper (T_{FH}) subset, a subset that is essential for B cell differentiation.

In conclusion, our data showed that HIV-2 is able to infect lymphoid tissue in a coreceptor-dependent manner, but that the replication cycle was impaired in lymphocytes at the transcriptional level. These findings on *in vitro* infection of lymphoid tissues by HIV-2 will provide new insights regarding HIV immunopathogenesis.

Keywords: Lymphoid organs, human tonsillar tissue, *in vitro* organ cultures, HIV-1 and HIV-2 infection.

Resumo

Os órgãos linfóides proporcionam respostas imunitárias contra patogénios e é onde estão a maior parte dos linfócitos existentes no corpo humano. Estes órgãos são os maiores reservatórios da infeção pelos vírus de imunodeficiência humano (VIH), no entanto o impacto da infeção é maioritariamente estudado no contexto do VIH-1. O VIH-2 representa uma forma atenuada da doença, caracterizada pela lenta diminuição de linfócitos $CD4^+$ e progressão para SIDA. Para além disso, o impacto na sociedade é muito menor que o do VIH-1. Contudo, o VIH-2 é um modelo único de ocorrência natural valioso para o estudo da patogénese do VIH. Por esta razão, compreender o impacto da infeção por VIH-2 em órgãos linfóides poderá levar a novas descobertas relativas à habilidade destes tecidos proporcionar respostas imunitárias. Neste projeto, investigámos o impacto do VIH-2 em tecido de amígdala humana, um órgão linfóide secundário. Culturas de amígdala foram infetadas com isolados primários de VIH-1 ou VIH-2 que usam ou o corecetor CCR5 (R5) ou o CXCR4 (X4). Demonstrámos que ambos os vírus são eficientes a infetar o tecido, tendo sido evidenciado por imunohistoquímica. Para além disso, demonstrámos que os linfócitos derivados da cultura de amígdala infetados por VIH-2 tinham uma quantidade de ADN viral integrado semelhante ao das infetadas por VIH-1. Surpreendentemente, a quantidade de mRNA e proteína Gag produzidos mostrou ser significativamente mais elevada na infeção do tecido pelo VIH-1 X4 comparativamente ao VIH-1 R5 e ao VIH-2, sugerindo que o vírus observado no tecido poderá estar relacionado com infeção de outras células, e que a transcrição do vírus X4 HIV-2 poderá estar alterada. No entanto, apesar da baixa replicação associada aos linfócitos observada no tecido infetado por VIH-2 X4, o impacto na viabilidade de linfócitos T $CD4^+$ e no compartimento de linfócitos T $CD4^+$ memória foi semelhante ao observado com o VIH-1 X4. No entanto, a infeção pelos vírus que usam o coreceptor CXCR4 resultou no aumento da expressão de Foxp3 nas células dos tecidos, uma molécula associada ao fenótipo regulador, e tal não se deveu a um aumento da proliferação destas células. Observámos ainda que o VIH-2 X4 depletou significativamente as células $Foxp3^+$ foliculares, persistindo preferencialmente linfócitos T reguladores $Foxp3^+$. Por último, observámos uma depleção dos linfócitos T foliculares por parte de todos os vírus. Em conclusão, os nossos dados mostram que o VIH-2 é capaz de infetar o tecido dependentemente do corecetor, mas o ciclo de replicação foi diminuído nos linfócitos a um nível transcricional. Estes resultados relativos à infeção de órgãos linfóides *in vitro* por VIH-2 permitirão novas descobertas sobre a imunopatogénese do VIH.

Palavras-chave: Órgãos linfóides, tecido humano de amígdala, culturas de órgão *in vitro*, infeção por VIH-1 e VIH-2.

Resumo Alargado

A síndrome de imunodeficiência adquirida (SIDA) é uma doença que já matou mais de 35 milhões de pessoas em todo o mundo[1]. Foi descoberta em 1981 como uma combinação de infeções oportunistas e tumores marcados pela redução de linfócitos $CD4^+$ circulantes[2]. Anos mais tarde, a SIDA foi associada à infeção de dois retrovírus, o VIH-1 e o VIH-2[3–6]. Os linfócitos $CD4^+$ são os principais alvos dos vírus devido à expressão de CD4 e dos corecetores CCR5 e CXCR4[7–9]. Existem outras células como os macrófagos e as células dendríticas que também expressam o recetor CD4, sendo por isso também alvos de infeção[10]. Ambos os vírus partilham mecanismos de replicação viral, modos de transmissão e consequências clinicas semelhantes, no entanto a progressão da doença ocorre mais lentamente na infeção por VIH-2 comparativamente à infeção por VIH-1[11]. Apesar da quantidade de ADN integrado nas células ser semelhante nas duas infeções[12], em indivíduos infetados com VIH-2 a depleção de linfócitos $CD4^+$ ocorre mais lentamente e a virémia permanece baixa ou indetetável[13]. O VIH normalmente infeta linfócitos $CD4^+$ ativados onde a replicação é mais rápida e eficiente[14]. Estudos prévios demonstraram que a depleção das células $CD4^+$ está diretamente relacionado com ativação imunitária em ambas as infeções[15]. No entanto, estes vírus também infetam linfócitos CD4+ com fenótipo de memória sem estarem ativadas[16,17], aliás este é considerado um grande reservatório de infeção e por isso muito estudado[18].

A maioria dos linfócitos que existem no corpo humano encontram-se em órgãos linfóides e desde muito cedo foi posto em questão se seriam reservatórios de infeção de VIH-1. Pantaleo, G. *et al.* (1991) descobriu de facto que os órgãos linfóides eram os maiores reservatórios da infeção por VIH-1. Mais tarde Jobe, O. *et al.* (1999) demonstrou que o mesmo ocorria na infeção por VIH-2. Desde então os órgãos linfóides têm sido muito estudados, pois são importantes para conhecer melhor os mecanismos de patogénese viral. Estes órgãos podem ser divididos em primários e secundários[9] e têm um papel importante na defesa contra patógenios[21] promovendo respostas imunitárias e mantendo as populações de linfócitos necessários para essas respostas[22]. Os centros germinativos nos tecidos linfóides são zonas onde os linfócitos B se diferenciam, com o auxílio dos linfócitos T foliculares, em plasmócitos, as células com capacidade de produzir anticorpos. A produção das células T foliculares é controlada a vários níveis desde o momento em que a célula T interage com células dendríticas até ao momento em que é guiada até ao centro germinativo e ganha as funções de célula T folicular[23]. Ao longo do processo de diferenciação, os linfócitos T ganham marcadores que

caracterizam as células T foliculares, tais como CXCR5 e PD-1. Estas células têm um fenótipo de memória[23–27].

Neste projeto, investigámos o impacto do VIH-2 no tecido de amígdala humana, um órgão linfóide secundário. Infetámos culturas de tecido de amígdala com VIH-1 ou VIH-2 que usam os corecetores CCR5 ou CXCR4. Demonstrámos que ambos os vírus são eficientes a infetar o tecido, o que foi evidenciado por imunohistoquímica. Para além disso, demonstrámos que os linfócitos do tecido infetados por VIH-2 possuem uma carga proviral semelhante aos linfócitos infetados por VIH-1, confirmando estudos anteriores[20]. Surpreendentemente, a quantidade de Gag mRNA e de proteína produzidos foi significativamente mais elevada na infeção do tecido pelo VIH-1 X4 comparativamente ao R5 VIH-1 e ao VIH-2, sugerindo que o vírus observado no tecido poderá estar relacionado com infeção de outras células, e que a transcrição do vírus X4 HIV-2 poderá estar alterada. No entanto, apesar da baixa replicação do VIH-2 X4 nos linfócitos do tecido infetado, o impacto na viabilidade de linfócitos $CD4^+$, e em particular, nos linfócitos $CD4^+$ memória, que mostram ser permissivos ao VIH[29,31,32], foi semelhante ao observado com o VIH-1 X4. Estes resultados são semelhantes aos obtidos por Schramm, B. *et al.* (2000) ao avaliar o impacto do VIH-2 no tecido linfóide primário e por Penn, M. L. *et al.* (1999) que demonstrou que o HIV-1 X4 diminuía consideravelmente os linfócitos $CD4^+$, em comparação com o VIH-1 R5.

O nosso estudo mostrou que havia uma tendência para a diminuição da proliferação de linfócitos isolados do tecido infetado por VIH-1 ou VIH-2. Este resultado não se encontra de acordo com o que está descrito em sangue de indivíduos infetados pelo VIH. Estes indivíduos infetados com VIH-1 ou VIH-2 com o mesmo nível de depleção de células $CD4^+$, mas com diferenças na virémia mostraram elevações nas frequências de linfócitos T ativados semelhantes[13,34]. O facto da carga proviral ser semelhante em TOCs infetadas com VIH-1 X4 e VIH-2 X4, bem como a elevada depleção dos linfócitos $CD4^+$, levou-nos a pensar que houvesse ativação imunitária e logo linfócitos a proliferarem. Por isso, este resultado poderá dever-se ao facto de usarmos um tecido fora do seu ambiente e por isso o número de células é limitado levando à sua exaustão.

Observámos também que a infeção por VIH-1 X4 e o VIH-2 X4 aumentava a frequência de células a expressar Foxp3, uma molécula associada com o fenótipo regulador, no tecido, no entanto a proliferação dos linfócitos T reguladores era diminuída, o que seria de esperar visto que a proliferação dos linfócitos $CD4^+$ totais também demonstrou estar diminuída. Anteriormente, já foi demonstrado que havia um aumento da frequência destas células no sangue de doentes infetados com o VIH-1 e o VIH-2[35] bem como nos

órgãos linfóide de doentes não tratados[36]. Observámos também o impacto da infeção nas células T reguladoras foliculares e mostrámos que os vírus VIH-1 e VIH-2 X4 diminuíam a frequências destas células, significando que as células que persistem não são as foliculares. Não há muita informação sobre a infeção por VIH destas células, no entanto Miles, B *et al.* (2015) demonstrou que a frequência das mesmas está aumentada na infeção por VIH-1.

Uma das razões que nos leva a investigar a infeção por VIH nos órgãos linfóides é devido ao papel que linfócitos T foliculares têm nestes tecidos. Como os linfócitos B são apenas capazes de sobreviver, expandir e diferenciarem caso encontrem um linfócito T folicular[38], estas têm impacto na produção de anticorpos. Estes linfócitos são muito estudadas na infeção por VIH-1[39–42], no entanto não existem estudos referentes ao VIH-2. Demonstrámos pela primeira vez que os VIH-2 têm o mesmo impacto que o VIH-1, diminuindo a frequência destas células, no entanto é mais significativo nos VIH X4. Kohler, S. L. *et al.* (2016) mostrou que o VIH-1 X4 tinha mais impacto nestas células que o VIH-1 R5, o que está de acordo com os nossos resultados. O facto de termos demonstrado que as células T foliculares expressam significativamente mais CXCR4, suporta que a infeção é dependente do corecetor. A população PD-1^{-}CXCR5^{+} foi diminuída nos tecidos pela infeção por VIH-1 e VIH-2 X4, comparado com o controlo. A expressão de CXCR5 leva a que as células migrem para o folículo das células B[23,24,43], e sem linfócitos B, a população PD-1^{-}CXCR5^{+} desaparece[26,44]. Durante a infeção por VIH-1 também foi sugerido que tanto a expressão de PD-1 como a de CXCR5 podem estar diminuídas, o que pode explicar os resultados anteriores[42]. Por outro lado, foi observado um aumento na frequência das células PD-1^{-}CXCR5^{-}, o que pode dever-se a preservação da população indiferenciada nas TOCs infetadas pelo VIH-1 e VIH-2.

Em conclusão, os nossos dados mostram que o VIH-2 infeta o tecido e que o seu impacto é dependente do coreceptor, mas que parece estar associado a um bloqueio transcricional da replicação. Estes resultados contribuem para um melhor conhecimento dos mecanismos de patogénese do VIH nos tecidos linfóides.

Agradecimentos

"Tenho a impressão de ter sido uma criança a brincar à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante dos meus olhos"

Isaac Newton, 1687

Uma folha de papel não chega para agradecer às muitas pessoas que estiveram comigo nesta brincadeira à beira-mar. Espero um dia conseguir dar um pouco do que elas me deram a outros.

Em primeiro lugar, quero agradecer à Dra. Ana Espada de Sousa por me ter aceite no seu laboratório, confiando em mim para este projeto, embora sabendo que os meus conhecimentos nesta área não eram muitos.

Às miúdas que me orientaram ao longo deste ano, que acima de tudo me deram umas belas asas para poder voar neste mundo lindo dos vírus. Helena e Cheila, muito obrigada por toda a simpatia e alegria que me proporcionaram ao longo deste ano. Trabalhar com alguém tão bem-disposta como vocês é meio caminho andado para o sucesso. Não podendo esquecer claro, todos os ensinamentos que me deram e discussões relativas ao trabalho que me fizeram dar uso ao meu ainda pequenino cérebro. Obviamente que também não me vou esquecer das belas horas passadas no P3 a ouvir Cidade FM com a Helena porque ali é que passa música *fixe* e com a Cheila a dançar ao som de *Justin Bieber*.

À juventude do laboratório com quem eu partilhei muito bons momentos, especialmente almoços. Foram essenciais para manter toda uma sanidade mental. À minha compincha destas andanças, Andreia. Obrigada por me ouvires a falar das minhas frustrações e me teres aturado ao longo deste ano. No fim disto tudo, temos de ir comer um grande mil folhas para comemorar e ter a nossa saída à noite. À Ana Rita um grande grande obrigado por tornares as minhas *imunos* lindas, sem ti esta tese não tinha tanta cor! E claro por toda a boa disposição que tornaram os meus dias mais alegres. À Ana Luísa, um grande obrigado pela disponibilidade e convivência que me proporcionaste. À Catarina, à Yummie e ao Henrique muito obrigada pelas palhaçadas, pelas conversas, pelos cafés e está claro pelos gelados. À Dona Alcinda e à Dra. Conceição, obrigada por me mostrarem que a idade é apenas um número, quem me dera a mim hoje com 24 anos ter o espírito jovem que vocês têm.

Nunca poderei esquecer de referir a Paula, a Adriana e o Russell. Apesar de não terem continuado comigo este caminho, tiveram um papel essencial no meu início no laboratório e acredito que ainda tinha muito para aprender com vocês.

Não posso deixar de referir as *facilities* do IMM que foram essenciais para a elaboração deste projeto. Um grande obrigado à Inês do BSL3 que esteve sempre disponível para nós dentro e fora do nosso pequeno laboratório, onde realizávamos as infeções. Um grande obrigado também à Andreia, Ana e Tânia do Laboratório de Histologia e Patologia Comparada, sem elas não teríamos TOCs para fazer o trabalho nem teríamos conseguido fazer as imunohistoquímicas e tirar conclusões sobre elas.

Claro que não me podia esquecer dos meus amores, já são muitos anos, mas eu espero que me continuem a aturar por muitos mais porque isto sem vocês não é a mesma coisa. À Inês Belo, ao Simão e ao Ruben, um sincero obrigado.

Não posso deixar de agradecer ao pessoal que Évora me deu, que ainda hoje me continuam a aturar como aturavam na minha cidade. À minha colega de casa e grande amiga, Denise, obrigada pelos magníficos jantares que fizeste, por me teres mostrado o arroz tufado. Peço desculpa, por todas as vezes que chegava a casa e me punha a desabafar sobre o meu dia. À Daniela pelas maluquices e por me tentares sempre levar para maus (bons) caminhos. E ao Wi, por ainda não teres batido com a cabeça e por continuares a aturar-me e a ouvir os meus desabafos científicos.

Um agradecimento especial à Ana *Cachuchóide*, que conheci há relativamente pouco tempo, mas foi muito fundamental nesta fase. Estiveste sempre lá quando eu estava num dia menos bom e conseguiste manter a minha sanidade mental. *Think like a proton*! À Inês também quero deixar um obrigado por teres estragado a minha dieta com aqueles croissants maravilhosos, eternamente grata.

Por último, mas claramente não menos importante. Aos meus pais, sem eles nada disto seria possível. Obrigada pelo apoio e por acreditarem em mim. Obrigada também por me ouvirem sempre eu queria falar do meu trabalho e embora não percebessem muito do que eu estava a explicar tentavam sempre pensar comigo sobre as minhas ideias. À minha irmã que me ouviu e ouviu e ouviu. Um muito obrigado! Obrigada por me aturares já há 19 anos e por me deixares partilhar contigo todas as minhas frustrações e alegrias. À Isabel, obrigada pelo carinho grande que sempre me deste e pela força que me transmites para nunca desistir.

Muito obrigada a todos!

Abbreviations

AIDS	Acquired immunodeficiency syndrome
APCs	Antigen-presenting cells
ART	Antiretroviral therapy
Az	Azide
Bcl-6	B cell lymphoma 6
Blimp-1	B lymphocyte-induced maturation protein-1
BSA	Bovine serum albumin
BSL3	Biosafety laboratory level 3
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CM	Complete medium
CXCR	CXC chemokine receptor
DCs	Dendritic cells
DNA	Desoxiribonucleic acid
dNTP	Deoxyribonucleotide trisphosphate
ECs	Elite controlers
Env	Envelope
FDC	Follicular dendritic cells
Foxp3	Forkhead-box transcription factor P3
FRCs	Fibroblastic reticullar cells
FVD	Fixable viability dye
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCs	Germinal centers
gp	Glycoprotein
H&E	Hematoxylin and eosin
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
ICOS	Inducible T-cel costimulator
IFNα	Interferon alfa
IgG	Immunoglobulin G

IL	Interleukin
IM	Infection médium
LMNCs	Lymph node mononuclear cells
LOs	Lymphoid organs
LT	Lymphoid tissue
LTNPs	Long-term non-progressors
MAbs	Monoclonal antibodies
MFI	Median fluorescence intensity
mRNA	Messenger RNA
NAbs	Neutralizing antibodies
NF-κB	Factor nuclear kappa B
NRS	Normal rat serum
OCT	Optimal cutting temperature compound
PBMCs	Peripheral blood mononuclar cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
Pha	Phytohaemagglutinin
qPCR	Quantitative PCR
RM	Reaction mix
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse transcription-quantitative PCR
SDB	Sample dilution buffer
SEM	Standard error of the mean
SG-PERT	SYBR Green I-based product-enhanced reverse trancriptase
SLOs	Secondary lymphoid organs
T_{FH}	T follicular helper cell
T_{FR}	T folicular regulatory cell
TNFα	Tumor necrosis factor alfa
TOCs	Tonsil organ cultures
Treg	T regulatory cell
TZ	T cell zone
UNAIDS	The Joint United Nations Programme on HIV/AIDS
VLB	Virus lysis buffer

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1 Background

1.1 HIV/AIDS

The acquired immunodeficiency syndrome (AIDS) is an infectious chronic disease caused by the extensively studied Human Immunodeficiency Virus (HIV). AIDS was first recognized in 1981[45–48] by the United States Centers for Disease Control and Prevention as a combination of opportunistic infections and tumors occurring in the setting of markedly reduced circulating $CD4^+$ T cell counts[2]. However, in 1983 Barre-Sinoussi, F. *et al.* determined that there was an association between a retrovirus and AIDS. Afterwards, Jay Levy's group established, by virological and epidemiological evidence, that HIV type 1 (HIV-1) was the cause of AIDS[4].

HIV type 2 (HIV-2) was only found in 1986, when Clavel and his group reported the isolation of a novel retrovirus originated from West Africa. HIV-2 was found to have a similar morphology but was an antigenically distinct virus able to cause AIDS[5,6].

Even though only in 1981 the world was confronted with a new epidemic when the first cases of AIDS were observed, the first positive HIV samples were collected from Congolese individuals in 1959[49]. Almost 40 years after the AIDS epidemics, this disease has already killed 35 million people worldwide[1] (Figure 1). Although there is still no cure for AIDS, since the identification of HIV as its causative agent, there have been impressive scientific advances in the development of effective antiretroviral drugs in order to improve the prognosis of HIV-infected individuals[45]. Antiretroviral therapy (ART) allows HIV-infected individuals to live longer and for that reason HIV infection has become a manageable chronic disease[50]. Nevertheless, it does not fully restore the immune system, and for that reason researchers are focused in obtaining a potential cure or even a vaccine that would allow the prevention of the disease[51].

Figure 1 - Global statistics from the UNAIDS 2015 report.[1]

1.1.1 The Human Immunodeficiency Virus

HIV belongs to the *Lentivirus* genus of the *Retroviridae* family[52] (Figure 2). Retroviruses carry their genetic information in the form of RNA[7]. The virus contains two copies of a single-stranded genomic RNA[46] and the enzymes required for replication events[53], all surrounded by a lipid bilayer membrane which is host derived. There are three types of proteins that allow the virus to establish an infection: the viral enzymes [reverse transcriptase, integrase and protease], the accessory proteins [Vpu (HIV-1), Vpx (HIV-2), Vif, Vpr, Nef, Rev and Tat] and the structural proteins [matrix, capsid, envelope proteins and nucleocapsid].

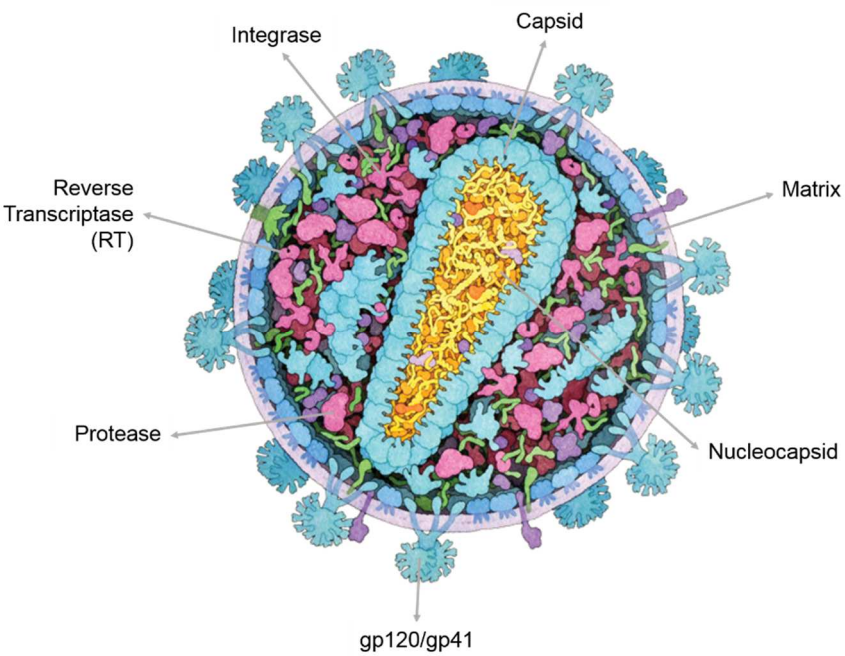

Figure 2 - Structure of HIV. HIV has two strands of RNA, 15 types of viral proteins, and a few proteins from the last host cell it infected, all surrounded by a lipid bilayer membrane. The virus enters the cell using the glycoprotein (gp) 120 and gp41 (HIV-1) and with the help of reverse transcriptase, protease and integrase, it is able to replicate inside the cell. Adapted from PDB 2002.

HIV-1 and HIV-2 have a similar structural and genomic organization, and they share 30-60% genetic similarity[55] (Figure 3). At the protein level, HIV-2 envelope glycoprotein has approximately 35% homology with HIV-1 and at the nucleic acid level, the viruses sequences have 57% homology[56,57]. HIV-2 was first identified when serum samples collected from West African individuals showed an unusual pattern on Western blot, with an absence of detectable antibody reactivity to HIV-1 envelope (*Env*) protein but the presence of reactivity to the more conserved HIV-1 Gag protein, as well as additional antibody reactivity directed toward the *Env* protein from Simian Immunodeficiency Virus (SIV)[57–59].

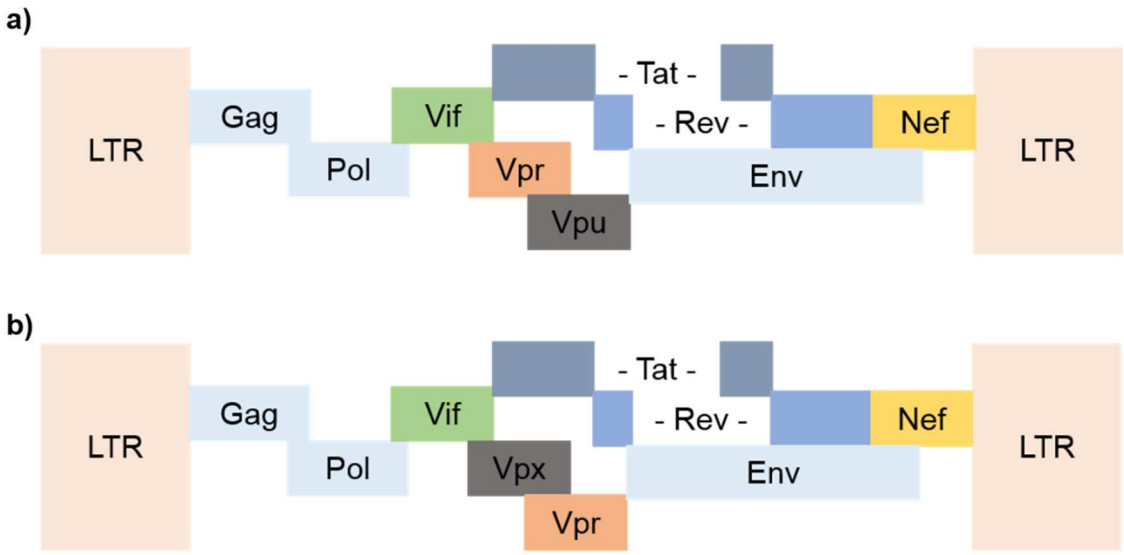

Figure 3 - Genetic organization of HIV. a) Genetic organization of HIV-1. b) Genetic organization of HIV-2. Even though they share a similar structure and genomic organization, they have some differences: HIV-1 expresses the Vpu gene, which is essential for the virus release, whereas HIV-2 expresses Vpx instead. Vpx has been extensively studied because of its relevance in macrophages infection. Adapted from Fanales-Belasio *et al.* (2010).

It was initially determined that the main immunological feature of HIV-infected individuals was the decrease in the number of circulating $CD4^+$ T cells and this feature rapidly became the hallmark of the disease[2]. $CD4^+$ T cells are the major HIV targets due to the high-affinity interaction between HIV envelope glycoproteins and the host cell-surface proteins: CD4, and CXCR4 or CCR5[7–9]. However, there are still other cells expressing the CD4 marker, such as macrophages and dendritic cells (DCs), which can be also infected by the virus[10].

In order to enter the host cell, both viruses use a membrane fusion mechanism[48] in which *Env* protein has an important role. *Env* protein is considered an entry machine because it binds to CD4, undergoes a series of conformational changes, fuses with the cell and delivers the viral core to its cytoplasm[61]. Following the entrance of the virus into the host cell, replication begins. Viral reverse transcriptase generates a DNA copy of the RNA viral genome, which is subsequently integrated into the target cells genome by HIV integrase. Once the host cell is activated, the integrated HIV provirus is transcribed. This instantly generates multiply spliced viral proteins: Nef, Tat and Rev. Rev is able to facilitate the export of unspliced viral mRNA into the cytoplasm, where it is cleaved by the HIV protease. The new viral RNA and viral proteins translocate to the cell surface to assemble into new immature virus forms that are released[51]. The replication cycle of HIV is shown in Figure 4.

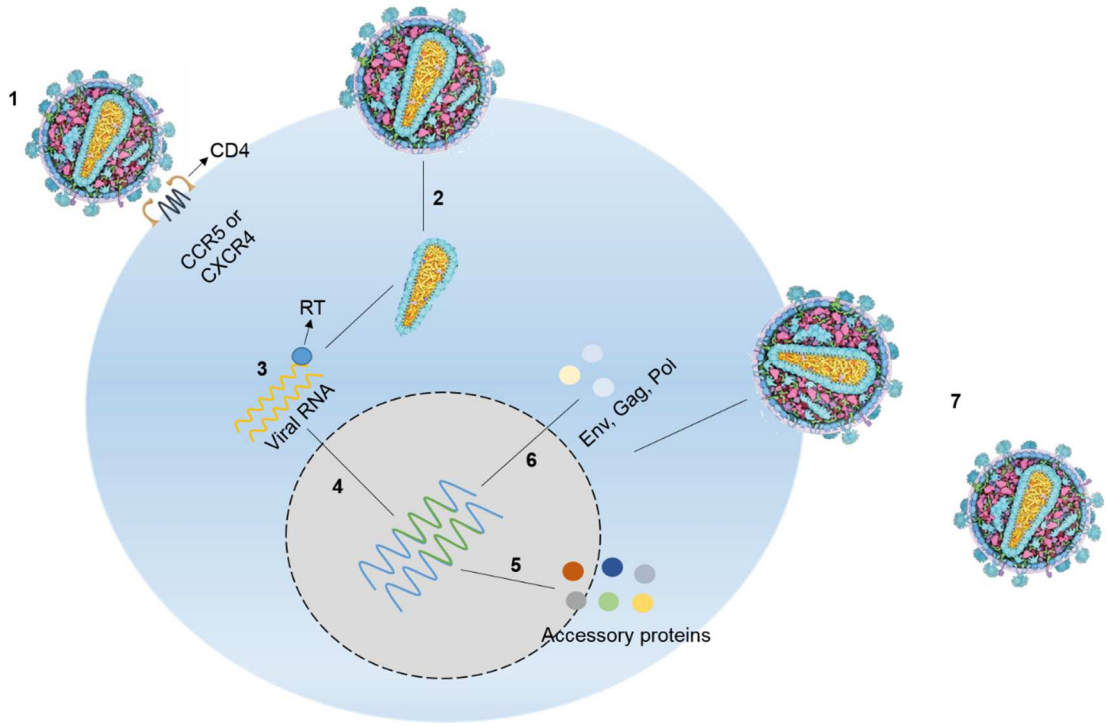

Figure 4 - Replication cycle of HIV. In order to enter the cell, HIV binds to the receptor CD4 and either the coreceptor CXCR4 or CCR5 (1). The virus fuses the host cell membrane and releases its genetic material (2). Following the entrance into the cell the viral reverse transcriptase transcribes its RNA strands into cDNA (3). Integrase is one of the viral enzymes which is able to integrate the cDNA into the host cell genome, being afterwards transcribed (4). This instantly generates multiply sliced viral proteins: Nef, Tat and Rev (5,6). Rev is able to facilitate the export of unspliced viral mRNA into the cytoplasm leading to the translocation of new viral RNA and viral proteins (Env, Gag, Pol) into the cell surface to assemble and then be released (7). Adapted from Rambaut *et al.* (2004).

1.1.2 HIV Pathogenesis

The hallmark of HIV infection is a progressive depletion of $CD4^+$ T cells in close association with progressive impairment of cellular immunity and increasing susceptibility to opportunistic infections[63]. Sousa, A. E. *et al.* (2002) showed that $CD4^+$ T cell depletion is directly related to immune activation in both HIV-2 and HIV-1 infections but only indirectly to plasma viral load. HIV mainly infects activated $CD4^+$ T cells[34] where viral replication is rapid and efficient[14]. One of the direct consequences of activation of the immune system is the secretion of pro-inflammatory cytokines, such as interferon alpha (IFNα), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 1 (IL-1) and interleukin 18 (IL-18), which can contribute to additional immune activation and apoptosis of immune cells[64]. HIV-1 proteins such as gp120, Tat, Nef and Vpu also play a role in driving the production of pro-inflammatory cytokines by directly inducing the activation of lymphocytes and macrophages[65]. Another consequence of T cell activation is the increase of NF-$_k$B levels[66]. This transcription factor is activated and it allows the virus replication which will promote immune activation and activate pro-inflammatory cytokines (IL-1β, IL-6 and TNFα)[65], serving a positive feed-back loop.

The activation of T cells implies their turnover and this is also a characteristic feature of HIV-1 and HIV-2 infections[65]. Sousa, A. E. *et al.* (2002) evaluated T cell turnover in HIV-1 and HIV-2 infected individuals with similar $CD4^+$ T cell counts and different viral loads, assessing the expressing of the nuclear factor Ki67. Ki67 is up-regulated in all cell cycle phases except G_0[67]. They found that there was an increased proportion of $Ki67^+$ cells within the CD4 subset. HIV-1 and HIV-2 patients present the same level of $CD4^+$ T cell depletion and exhibit similar elevations in the frequencies of activated and cycling T cells despite large differences in viremia, indicating that CD4 depletion is more related to the overall activation and turnover of T cells than to the rate of virus replication[13]. Moreover, the majority of the cycling cells had a memory phenotype in both infections[13].

$CD4^+$ T cells can be divided phenotypically into naïve and memory subsets[32,68]. $CD4^+$ or $CD8^+$ T lymphocytes emigrating from the thymus express the CD45RA antigen, being immunologically naïve[69]. After challenged with an antigen in the periphery, they convert into a CD45RO phenotype which is characteristic of memory T cells[69]. Memory cells comprise a greater portion of latent proviral infection than do naïve cells[18]. After entry into the T cell, HIV may establish a persistent or a latent form of infection[48]. When T cells remain in the G_0 phase of the cell cycle, they persist in a resting state[70]. The majority of HIV-infected resting $CD4^+$ T cells *in vivo* exhibit a memory phenotype[16,17], but those are non-permissive for the virus replication[48,70,71], representing one of the major long-lived reservoirs *in vivo* for HIV infection[45,72,73].

Regulatory T cells (Tregs) actively down-regulate immune responses[74]. They are responsible for the maintenance of homeostasis of the immune system that limits the magnitude of effector responses and allows the establishment of immunological tolerance[38]. These cells express the transcription factor Foxp3 which is considered the master regulator of their differentiation[38,75,76]. Because HIV-associated immune activation is a strong predictor of disease progression, Tregs have been studied in this context. Tregs are detected in peripheral blood and in mucosal and lymphoid tissues of HIV-infected patients and may contribute to immune deficiency and/or, conversely, be beneficial through a decrease in immune activation, depending on the state of the disease[77]. On one hand, Tregs can be infected more susceptibly by HIV-1 X4 strains[78] which is associated with immune activation[74]. On the other hand, in untreated progressive HIV-1 and HIV-2 infection, despite an overall decline of $CD4^+$ T cells, Treg frequencies are increased[79]. Furthermore, there is an increase in Ki67 expression in circulating Tregs from untreated, chronically infected patients prior to undergoing ART[80,81], as well as from HIV-2-infected patients[35]. Regulatory T cells have been

associated with several roles in HIV infection, which may occur at different times during the infection process and may be affected by ongoing therapy[38].

$CD4^+$ T cells represent the most drastically affected lymphocyte cell type after HIV infection, however other leukocyte subsets are also altered. Indeed, cell cycle perturbations, apoptosis, immune senescence and altered functionality among $CD8^+$ T cells, B cells, and innate immune cells have also been described[64].

The rate of HIV progression to AIDS can be determined by a multitude of factors, including immunological, genetic, viral and environmental. During HIV infection, the establishment of immune activation and inflammation involve several mechanisms that are either directly or indirectly related to viral replication[65]. The mechanisms that have been proposed to cause HIV-associated immune activation include the direct effect of specific virus gene products (Env, Nef and Tat); the innate and adaptive immune responses to the virus; an ineffective regulation of normally generated antiviral immune responses; bystander activation of T and B lymphocytes caused by an increased level of production of pro-inflammatory cytokines; the presence of clinical or subclinical co-infections; and more recently the preferential infection of central memory $CD4^+$ T cell as a factor responsible for concentrating the bulk of antigenic load in central lymphoid tissues[82–84].

1.1.3 HIV-1 vs. HIV-2 disease progression

HIV-1 and HIV-2 have many similarities including intracellular mechanisms of replication, modes of transmission and clinical consequences. However, the progression to immunodeficiency occurs more slowly in HIV-2 infection compared with HIV-1[11]. In HIV-2 patients CD4 depletion occurs at a much slower rate than in HIV-1-infected individuals, and plasma viremia remains low to undetectable, which might be linked to the reduced rates of both vertical and horizontal HIV-2 transmission[13]. However, the levels of proviral DNA have been shown to be similar in both infections[29]. The slower rate of CD4 depletion and the viremia profiles led HIV-2 patients to be defined as long-term non-progressors (LTNPs) or elite controllers (ECs)[57]. On the other hand, HIV-2 infection is still a progressive disease. There are many people infected with HIV-2 who develop AIDS, indicating a similar clinical outcome despite the slower time course of HIV-2 infection[85].

Both HIV-1 and HIV-2 infected individuals have access to ART which suppresses HIV replication and increase the $CD4^+$ T cell counts[86]. Even though highly active antiretroviral therapy (HAART) is effective in HIV-1 infected patients, it doesn't show the same effect in HIV-2 infected patients, as Mullins, C. *et al.* (2004) demonstrated. The authors studied 10 HIV-2 infected individuals treated in the United States with very low viral load

expecting that HAART would suppress the viral load. However, HAART regiments failed to do so. HIV-2 infected people face many difficulties regarding ART therapy compared to HIV-1 infected individuals. First, the viral load is not a good tool to monitor treatment, and second, since there are no clinical trials targeting the HIV-2 infected population, there are no guidelines for therapy and some antiretroviral drugs designed against HIV-1 are not effective in inhibiting HIV-2 propagation[88].

1.2 Infection of Lymphoid Tissues by HIV

In the early days, PBMCs were used to study HIV-infected individuals since this compartment is readily accessible. However, the majority of the body's lymphocyte pool resides in lymphoid organs (LOs)[89]. Although, it was estimated that 2% of total lymphocytes in the body circulate in the blood[90], more recently Di Mascio, M. *et al.* (2006) showed that the peripheral blood only contains between 0.3% and 0.5% of total $CD4^+$ T cells in the body, with the remaining lymphocytes residing in LOs. Despite the consistent observation $CD4^+$ T cell depletion in HIV infection, there was inconsistency in the different magnitudes of quantitative depletion of those cells vs. low frequency of infected cells found in the PBMCs of infected individuals[19,92–95]. When AIDS epidemic started, lymph node biopsies were performed for diagnostic purposes in individuals who were observed to have immunodeficiency associated with generalized lymphadenopathy[96]. Moreover, Barre-Sinoussi, F. *et al.* (1993) isolated HIV-1 from a lymph node. So, Pantaleo and his colleagues compared the presence of HIV-1 DNA between PBMCs and lymph nodes from infected individuals and demonstrated that lymphoid organs function as major reservoirs for HIV-1 infection. In order to determine whether lymphoid organs were also a reservoir for HIV-2 infection, Jobe, O. *et al.* (1999) compared proviral DNA between PBMCs and lymph node mononuclear cells (LNMCs) and demonstrated that lymph nodes are also a reservoir for HIV-2 infection. The delineation of the role of lymphoid organs in HIV infection[97] has facilitated the understanding and identification of multiple mechanisms involved in the pathogenesis of HIV infection[98].

1.2.1 Immunology of Lymphoid Organs

LOs provide an optimal environment for defense against the invasion of pathogens at any site of the body[21], promoting immune responses and maintaining normal sized populations of the principal players: T cells and antigen-presenting cells (APCs), such as B cells[22] (Figure 5). LOs can be classified functionally into primary lymphoid organs which provide appropriate microenvironments for the development and maturation of lymphocytes; and secondary lymphoid organs where lymphocytes can interact effectively with the antigen[7].

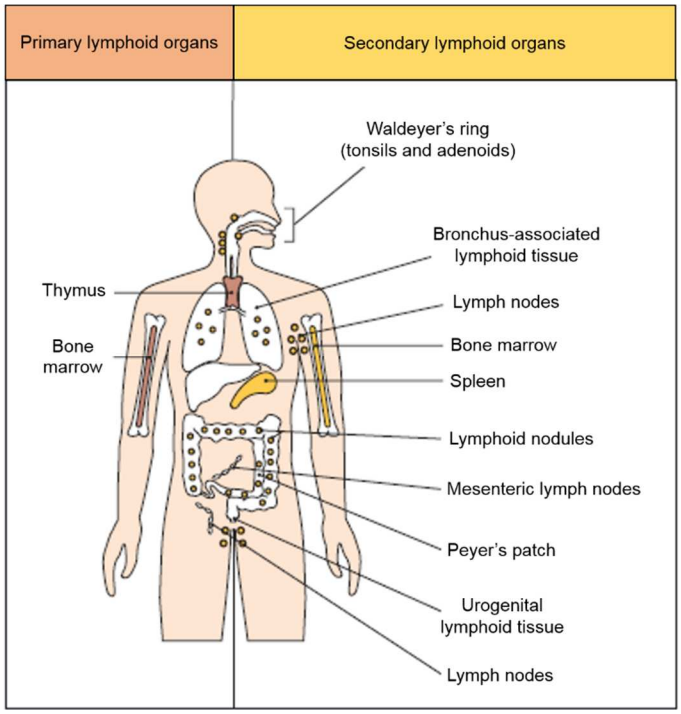

Figure 5 - Lymphoid organs. Lymphoid organs are divided into primary and secondary lymphoid organs. These organs are very important for the immune system. The largest part of the lymphocyte development occurs in the primary lymphoid organs such as the thymus and bone marrow. The secondary lymphoid organs are the place where the adaptive immune response begins. Tonsils, the lymph nodes and the spleen are some examples of secondary lymphoid organs.[9]

Cells of the T and B cell lineages migrate from the primary LOs to function in the SLOs[9]. The cells are guided by chemotactic gradients, where they interact with APCs, receive survival signals and initiate antigen-specific immune responses[99]. The survival of T cells depends on the niche in which they live at different stages of their differentiation [22,100,101]. The paracortical T cell zone (TZ), where 98% of $CD4^+$ T cells in the human body normally reside, is one such niche[102]. It has been shown that there is a gradual and progressive deposition of collagen within the TZ of secondary lymphoid tissues (LTs) in HIV-1 infection[103]. Naïve T cells within secondary LTs rely on the interaction with the fibroblastic reticular cell (FRC) network in the T cell zone to supply factor such as IL-7 for their survival[22,100,104,105]. The deposition of collagen disrupts migration and access of $CD4^+$ T cells to cytokines such as IL-7[103]. The depletion of $CD4^+$ T cells leads to the weakness of the immune system, due to progressive immunodeficiency, restricting the ability of infected individuals to adequately defend against infections[106]. Under optimal circumstances in previously untreated individuals, HAART can suppress replication of HIV-1 to undetectable levels in the bloodstream[107]. Nevertheless, populations of $CD4^+$ T cells remain depleted by as much as 50% in secondary lymph nodes (LNs) and gut associated lymphoid tissue (GALT) [108]. Zeng, M. *et al.* (2012) have shown that the recovery of T cells within lymphoid tissues is limited even after starting the antiretroviral

therapy. The investigators mentioned that the restoration of the FRC is a slow process, notwithstanding starting HAART in the acute phase of the infection since there is a reconstitution of FRCs but not to the level of uninfected population.

The B cells' niche is another compartment in lymphoid organs which is anatomically separated from the T cells niche. B cells interact with antigens on the follicular dendritic cell (FDC) networks[103] and when stimulated they migrate and proliferate within follicles forming distinctive germinal centers (GCs)[106]. GCs emerge from secondary lymphoid organs follicles after encounter with a T cell-dependent antigen[109]. Activated T and B cells migrate to the T-B zone border where they interact with each other[44] and that process leads to the entrance of B cells into follicles with the help of T cells, forming the GC[110]. This interaction is also important for the survival, expansion and differentiation of B cells[109] into plasma cells and memory B cells[111]. These cells have been described as crucial for the development of antigen-specific B cells within GC[112]. They were first known as follicular B helper T cells, but nowadays they are known as follicular helper T cells (T_{FH})[113].

T_{FH} were first identified as a subset of $CD4^+$ T cells isolated from human tonsils[24,43]. These cells are characterized by high-levels of surface expression of the receptor CXCR5, of programmed death-1 (PD-1) and of the inducible costimulatory (ICOS), as well as by expression of the transcription factor B cell lymphoma 6 (Bcl-6)[24,43,114]. The differentiation into T_{FH} cells begins when naïve $CD4^+$ T cells encounter dendritic cells (DCs) with an increase of Bcl-6[26] within the T cell zone of SLOs[23,113]. The migration of T cells away from the T zone into the T-B border, where they meet cognate B cells, is induced by the upregulation of CXCR5 (homing receptor to B cell zone) and downregulation of CCR7 (homing receptor to T cell zone)[23,24,43]. Once T cells encounter cognate B cells, stable T-B conjugates are formed, PD-1 expression increases[26] and they move together into the GC[23]. Only B cells that pick up antigens and present those to cognate T_{FH} cells survive, expand, and undergo differentiation[23]. They regulate B cell responses through the secretion of cytokines such as interleukin-21 (IL-21) which is capable of modulating B cell differentiation and proliferation[113,115].

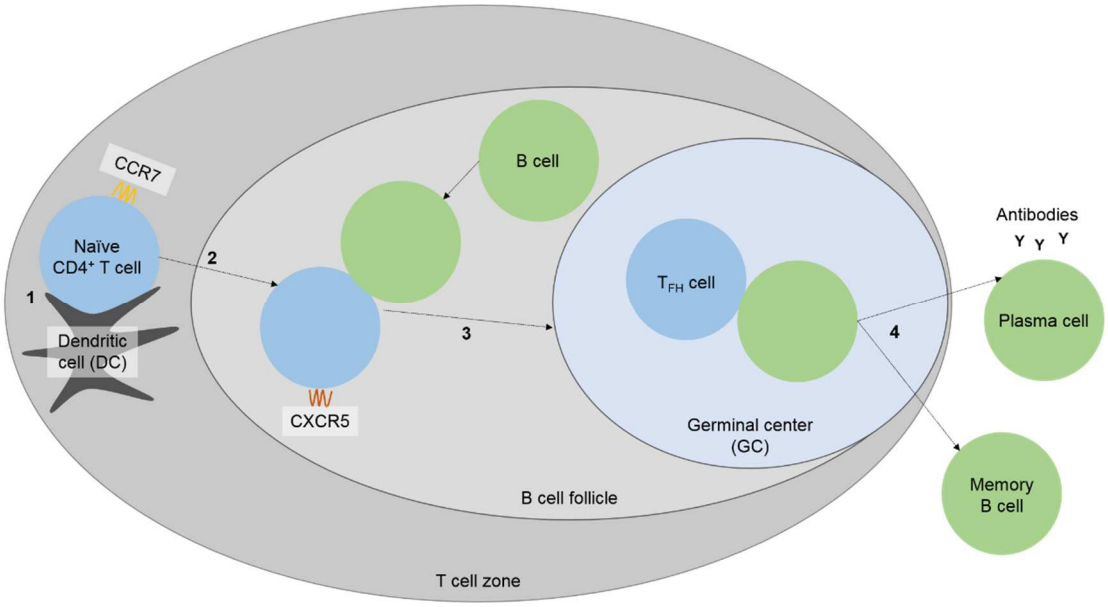

Figure 6 - T_{FH} differentiation. The differentiation into T_{FH} cells begins when naïve CD4⁺ T cells encounter dendritic cells (DCs) within the T cell zone of SLOs (1). The migration of T cells away from the T zone into the T-B border, where they meet cognate B cells, is induced by the upregulation of CXCR5 (homing receptor to B cell zone) and downregulation of CCR7 (homing receptor to T cell zone) (2). Once T cells encounter cognate B cells, stable T-B conjugates are formed, and they move together into the GC (3). Only B cells that pick up antigens and present those to cognate T_{FH} cells survive, expand, and undergo differentiation (4).[116]

It has recently come to light that B-cell follicles contain a novel subset of regulatory T cell (Treg), termed follicular regulatory T cells (T_{FR})[117–119]. Like T_{FH} cells, T_{FR} cells also express high levels of CXCR5, ICOS and PD-1[117–120]. Furthermore, up to 75% of these cells express high levels of Blimp-1 while still co-expressing Bcl-6 [121]. In T_{FH} cells, Blimp-1 and Bcl-6 play central but opposing roles in their differentiation, with Blimp-1 being down-regulated[122]. T_{FR} and T_{FH} share differentiation and regulation mechanisms[38] however they have opposing roles in regulating immune responses: whereas T_{FR} cells potently suppress humoral immune responses, T_{FH} cells stimulate them[117–120]. The role of T_{FR} in the immunopathogenesis of HIV infection is poorly understood, however there are some data that indicate that viral entry was necessary to promote T_{FR} expansion in HIV infection[37].

1.2.2 HIV Infection of Germinal Centers

Since T_{FH} cells have a key role in the production of antibodies and the main goal of researchers is to find a vaccine that could prevent the infection, they have been extensively studied in the HIV context. Studying both infections in lymphoid organs may give us new insights to achieve cure.

In HIV-infected individuals, T_{FH} cells have shown to contain the highest percentage of CD4⁺ T cells harboring HIV DNA and were the most efficient in supporting productive infection *in vitro*[40]. They are required for the induction of high-affinity antibody responses

and the formation of long-lived B cell memory, and deregulation of B cell function causes a progressive dysfunction of the humoral immune response[114,123]. Lindqvist, M. *et al.* (2012) showed that even though there is an expansion of T_{FH} during chronic HIV-1 infection, there is a profound skewing of the B cell compartment toward GC B cells and plasma cells and a corresponding reduction in the memory B cell population in the lymph nodes. Memory B cells are responsible for a rapid and specific antibody response on a second encounter with an antigen and these are known to be severely impaired in both HIV-1 and HIV-2 infections, in association with $CD4^+$ T cell depletion[124]. In acute and early HIV-1 infection and then in later stages of infection, B cell follicles become hyperplastic leading to the follicles to involute and lyse[108]. In isolated PBMCs from HIV-2-infected individuals a major loss of both switched and unswitched memory B-cells was observed which was not recovered by ART, despite the reduced amount of circulating virus[125]. Cubas, R. *et al.* (2013) co-cultured B cells and T_{FH} in order to evaluate the levels of IgG production in HIV-1 infection and reported a severe reduction of the levels compared to the control. T_{FH} were not able to provide adequate B cell help, leading to a failure in antibodies production and consequently to a diminished response to new immunization regarding HIV-1-infected individuals[39]. Numerous B-cell perturbations appear during HIV-1 infection and although some of these defects are improved by ART, B-cell responses to HIV specific antigens are diminished[126]. A study with children receiving ART showed that the function and phenotype of T_{FH} cells are impaired during HIV-1 infection which leads to the weakness of the humoral immunity[41].

2 Objectives

Lymphoid organs are known to provide the environment for immune responses to occur and are also believed to be the major reservoir of HIV infection. HIV-2 constitutes an attenuated model of HIV disease.

The overall objective of this project was to compare the impact of HIV-1 and HIV-2 infections in SLOs.

Our specific aims were:

1. To assess the infection and replication of HIV-2 in human tonsillar tissue, a secondary lymphoid organ, in comparison to HIV-1 infection;
2. To determine the impact of HIV-2 in cell viability, $CD4^+$ T cells and the memory $CD4^+$ T subset;
3. To analyze the importance of coreceptor usage for HIV-2 infection;
4. To understand the effect of infection in Treg cells and follicular T regulatory cells;
5. To address the impact of HIV-2 in T follicular helper cells.

3 Methodology

3.1 Samples

Buffy coats were obtained from healthy donors at the Instituto Português do Sangue, Lisboa, Portugal, and tonsil specimens were obtained from routine pediatric tonsillectomy at the Hospital de Santa Maria, Lisboa, Portugal, after parent's written informed consent. The study was approved by the Ethical Board of the Faculty of Medicine of the University of Lisbon.

3.2 PBMC Cultures

PBMCs were obtained from buffy coats of healthy donors through a Ficoll-Paque Plus (GE Healthcare) density gradient. First, 15mL of blood were diluted in an equivalent volume of PBS, and 15mL of Ficoll were added to the bottom of the tube. After centrifugation at 2000 rpm for 20 minutes, the Ficoll ring containing the PBMCs was placed in a 50mL falcon. Cells were washed twice with PBS by centrifuging at 1500 rpm for 15 minutes and then at 1200 rpm for 10 minutes, and counted in acetic acid 1N using a Neubauer chamber. PBMCs were cultured at $2x10^6$ cells per mL in complete medium (CM) (Rosewell Park Memorial Institute [RPMI; Gibco] 1640 suplemented with 10% fetal bovine serum [FBS; Sigma], 2mM L-Glutamin [Gibco], 100U/mL Penicillin/Streptomycin [Gibco] and 50µg/mL Gentamicin [Gibco]) stimulated with Phytohaemagglutinin (Pha; Sigma) at 37ºC/5%CO_2. After 2 to 3 days the medium was changed into CM plus interleukin-2 (IL-2; from Maurice Gately, Hoffmann-La Roche Inc., through the NIH AIDS reagent Program) at 10U/mL, which was replaced every 2 to 3 days.

3.3 Infection of PBMCs for Viral Stock

The infection of PBMCs for viral stock was performed at the BioSafety Laboratory Level 3 (BSL3). Five million cells were incubated with 1mL of highly concentrated virus (Table 1) for 1h30-4h. Afterwards, infection medium (IM) (CM plus IL-2 at 10U/mL and 3µg/mL polybrene [Sigma]) was added and PBMCs were cultured at $2x10^6$ cells/mL at 37ºC/5%CO_2. At day 1 of infection $10x10^6$ cells were added to each culture. After three days 2/3 of the supernatant were replaced, followed by the addition of $20x10^6$ cells. The supernatant removed was placed at -80ºC. This process was repeated every three days. On the 20th day of production the entire content of the culture flask was centrifuged at 1600rpm for 5 minutes. The supernatant was saved at -80ºC, while the cells were further washed with PBS at 2500rpm for 5 minutes and saved as dry pellets at -80ºC. At the end of the culture all supernatants saved throughout production were thawed, pooled and divided into 1, 3 or 15mL aliquots.

Table 1 - R5- and X4- tropic HIV-1 and HIV-2 primary isolates produced.

Primary Isolates		
Virus	***Major coreceptor used***	***Source***
HIV-1 $_{92US660}$	R5	NIH[a]
HIV-1 $_{92HT599}$	X4	NIH[a,b]
HIV-2 $_{60415k}$	R5	NIH[a,c]
HIV-2 $_{20.04}$[d]	X4	Nuno Taveira

[a]From the Multicenter AIDS Cohort Study, NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.
[b]Provided by Neal Halsey.
[c]Provided by Feng Gao and Beatrice Hahn.
[d]Previously represented as PTHCC20/2004[127] and 19/2004[128].

3.4 Quantification of the Viruses by SYBR Green Product-Enhanced RT (SG-PERT)

Viruses were quantified through the measurement of reverse transcriptase (RT) activity using the SG-PERT assay[129,130]. Virus lysis was performed at the BSL3 by adding 5µL of each virus supernatant to 5µL of 2X virus lysis buffer (VLB) (0.25% Triton X-100 [Sigma], 50mM potassium chloride [KCl; Sigma], 100mM Tris-chloride [Tris-HCl] buffer pH7.4 [Sigma], 40% Glycerol [Sigma] and water for molecular biology [Sigma]) containing 2µL of Ribolock RNAse inhibitor (Fermentas). After incubation for 10 minutes at room temperature, 90µL of 1X sample dilution buffer (SDB) (50mM ammonium sulfate [$(NH_4)_2SO_4$); Sigma], 200mM KCl [Sigma], 200mM Tris-HCl buffer pH8.3 [Sigma] and water for molecular biology [Sigma]) were added. For the real time PCR reaction, 10µL of lysed virus supernatant were added to 10uL of 2X reaction mix (RM) (2X SDB, 10mM magnesium chloride [$MgCl_2$; Sigma), 0.2mg/mL bovine serum albumin [BSA; Isaza], 400µM dATP [Invitrogen], 400µM dGTP [Invitrogen], 400µM dTTP [Invitrogen], 400µM dCTP [Invitrogen], 1µM forward primer [Table 2; Invitrogen], 1µM reverse primer [Table 2; Invitrogen], 7pmol/mL MS2 RNA [Roche], 1:10000 SYBR Green I [Invitrogen] and water for molecular biology [Sigma]) containing TrueStart HotStart Taq DNA (Thermo Scientific) in a 96 well plate. In parallel a standard curve using defined RT concentrations (5000pg/mL, 1000pg/mL, 200pg/mL, 40pg/mL, 8pg/mL and 1.6pg/mL) and treated the same way as the samples was run. Negative controls included the RM plus H_2O, as well as SDB complemented with RM and VLB. Reaction was performed at ABI 7500 Fast (ThermoFisher Scientific) using the following protocol: 30 minutes at 33ºC; 2 minutes at 95ºC; 40 Cycles of 5 seconds at 95ºC, 5 seconds at 60ºC, 15 seconds at 72ºC, 25 seconds at 82ºC (acquisition); and melting curve.

Table 2 - Primers used for the SG-PERT reaction.

Primers	Sequence
Forward Primer	TCCTGCTCAACTTCCTGTCGAG
Reverse Primer	CACAGGTCAAACCTCCTAGGAATG

3.5 Determination of the Viruses Infectivity

Infectivity of the viruses produced was confirmed by infecting activated PBMCs purified from the blood of three different volunteer healthy donors together. 3.5 million PBMCs were infected with 1050pg of RT of the different viruses produced (Table 1). The viruses were centrifuged at 50.000rpm for 30 minutes, the supernatant removed and ressuspended in 200µL of IM and added to the cells previously distributed in 500µL of IM in a 48 well plate. Cells were incubated at 37ºC/5%CO_2 for 4 hours. Next, cells were washed with PBS and ressuspended at 2.0x10^6 cells/mL. Finally, the infected cells were distributed infected cells in two 24 well plate and were incubated for 7 days at 37ºC/5%CO_2. PBMCs in IM only were used as negative control.

3.6 Tonsil Organ Cultures (TOCs) Optimization

After being collected, the tonsil specimens were washed and sectioned into quadrants, one of which was further divided into two similarly-sized portions. One of these was frozen in an Eppendorf containing optimal cutting temperature compound (OCT, VWR) and snap-frozen in liquid nitrogen. The other portion was fixed in formalin (Enzifarma) and then embedded in paraffin. The remaining tissue was cut in blocks of 1-8mm^3 using tweezers and a scissor in a Petri dish. A maximum of 7 blocks was placed over Millicell organotypic inserts (Millipore) placed in 1250µL of TOCs CM (RPMI1640 [Gibco] supplemented with 15% FBS, 2mM L-Glutamin [Gibco], 100U/mL Penicillin/ Streptomycin [Gibco], 1X non-essential aminoacids [NEA; Gibco], 1mM sodium pyruvate [Gibco] and 50µg/mL Gentamicin [Gibco]) and were incubated at 37ºC/5%CO_2.

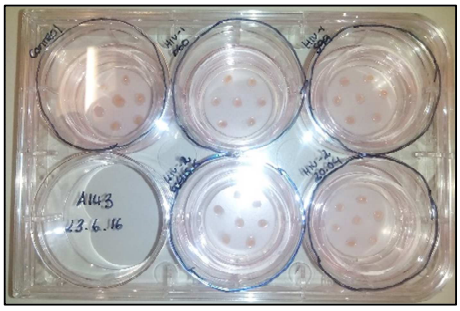

Figure 7 - TOCs cultured in Millicell organotypic inserts (Millipore) placed in TOCs CM.

3.7 TOCs Infection with HIV-1 and HIV-2

The infection of TOCs by HIV-1 and HIV-2 was performed at a BioSafety Laboratory Level 3 (BSL3). TOCs were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates (Table 1). The viruses were concentrated at 50.000rpm for 30 minutes, resuspended in 38µL of TOCs CM and 5µL of concentrated virus (or TOCs CM as negative control) were added to each TOC (Figure 7). TOCs were cultured for seven days at 37ºC/5%CO_2 with replacement of one third of the TOC CM every 2-3 days.

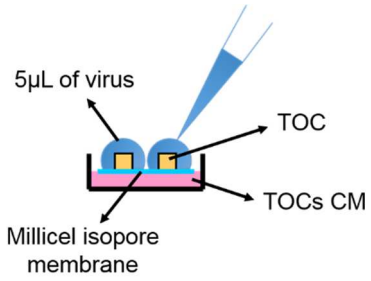

Figure 8 - Illustration of TOCs infection by HIV.

At the end of culture, two TOCs were embedded in paraffin after preservation in 4% formaldehyde to assess infection and tissue structure by immunohistochemistry. Additionally, five TOCs were mashed and cells were used to assess the tissue cell phenotype by flow cytometry, and to save cell pellets at -80ºC. Supernatant was also saved at -80ºC.

3.8 Flow Cytometry

Surface staining was performed for 20 minutes at room temperature and always included Fixable Viability Dye (FVD; eBiosciences) for dead cell exclusion. Cells were fixed, permeabilized and stained using an intracellular staining kit (eBioscience), according to the manufacturer's instructions. The anti-human MAbs used were (clone is described in parenthesis): CD3 (OKT-3), CD8α (RPA-T8), CD19 (HIB19), PD-1 (MIH4), CD4 (RPA-T4 and OKT-4), CD45RA (HI100), CD8α (SK1), CXCR4 (12G5), Ki67 (BS6) and FoxP3 (PCH101) from eBiosciences; CXCR5 (51505) from R&D Systems; CD25 (2A3), CCR5 (2D7), and CD3 (UCHT1) from BD Biosciences; CD45RA (HI100) from Pharmigen; CXCR5 (J252D4), CD45RO (UCHL1) and CD4 (OKT-4) from Biolegend. Gag protein production was quantified using KC57 (Beckmann Coulter), an antibody that detects both HIV-1 and HIV-2 Gag[131,132]. Cells were acquired at a LSRFortessa cell analyzer (BD Biosciences) and data were analyzed using FlowJo Software (TreeStar).

3.9 RNA/DNA Purification

RNA and DNA were extracted from infected TOCs cell pellets, using the ZR-Duet DNA/RNA MiniPrep kit (Zymo research), which allows the extraction of DNA and RNA at the same time. Cells were disrupted with DNA/RNA lysis buffer and the homogenized lysate transferred into a Zymo-spin IIIC column placed in a 2mL collection tube and centrifuged at 12000g for 1min. The Zymo-spin IIIC column was place in a new 2mL collection tube and stored at room temperature for later DNA purification. The flow-through was used for RNA purification adding initially 1,6 volume of 100% ethanol and mixing well with a pipette. The homogenized was transferred into a Zymo-spin IIC column in a collection tube and centrifuged at 12000g for a minute. The flow-through was

discarded and 400µL of RNA Wash Buffer was added to be centrifuged for 30 seconds at 12000g. The flow-through was discarded and 80µL of DNaseI Reaction Buffer Mix (1U/µL of DNaseI and 75µL of DNA Digestion Buffer) was added to each sample and kept at room temperature for 15 minutes. Afterwards, 400µL of RNA Prep Buffer was added and centrifuged at 12000g for 1 minute. The flow-through was discarded and 700µL of RNA Wash Buffer was added. The centrifugation lasted 30 seconds at 12000g and the step of washing with 400µL of RNA Wash Buffer was repeated after discarding the flow-through. The column was then placed in a new 2mL collection tube and centrifuged at full speed for 2 minutes so that the RNA was eluted in 25µL RNase-free water in a 1,5mL RNase free tube. To elute de RNA, the homogenized is centrifuged for a minute at 10000g twice. In order to extract DNA, to the Zymo-spin IIIC column that was placed into a new 2mL collection tube earlier was added 400µL of DNA Prep Buffer. The homogenized was centrifuged for 3s at 12000g and the flow through was discarded. 700µL of DNA Wash Buffer were then added and the column centrifuged for 30s at 12000g. After one more wash with 400µL of DNA Wash Buffer and centrifugation at 12000g for 30s the column was placed into a new 2mL collection tube and centrifuged at full speed for 2min. Finally, the column was placed in a 1.5mL collection tube and 50µL of RNase-free water added. After 5min incubation at room temperature the column was centrifuged for 30s at top speed to elute the DNA. The column was then reloaded with the eluent and centrifuged. DNA and RNA were quantified using a Nanodrop 2000 Spectometer (ThermoFisher Scientific). The DNA was saved at -20ºC and the RNA was saved at -80ºC.

3.10 Total viral DNA Quantification

Total viral DNA was quantified using quantitative PCR (qPCR). Two sets of primers formely designed and validated in the lab were used. In order to quantify total viral DNA in number of Gag copies per million cells, both Gag and CD3 were amplified in the same sample. In parallel, standard curves containing a known number of copies (10^7 to 5copies/5µL) were run using plasmids containing both HIV-1 or HIV-2[132] Gag and CD3. Table 3 and Table 4 show the PCR reaction mixes, PCR conditions and the primers and probes used in the process. The quantification was performed in ABI 7500 Fast (ThermoFisher Scientific).

Table 3 - Primers and probes used in qPCR to quantify total proviral DNA.

Primers and Probes			Brand
HIV-1 Gag	*Primer F5 (FW1Bgag5)*	CGAGAGCGTCAGTATTAAGC	Invitrogen
	Primer R5 (Rev1Bgag5)	AGCTCCCTGCTTGCCCATAC	
	Probe:	5'-FAM-CCCTGGCCTTAACCGAATT-MGB	Alfagene
HIV-2 Gag	*Primer F (F2)*	CGCGAGAAACTCCGTCTTG	Invitrogen
	Primer R (Gag2R1)	GCTGCCCACACAATATGTTTTA	
	Probe:	5'- FAM-CCGGGCCGTAACCT-MGB	Alfagene
CD3	*Primer F2.1*	AGGGCAAAATGGAGGCTCTTA	Invitrogen
	Primer R2.2	TCTCCTCCATGGGACACTGTT	
	Probe:	5'- VIC-CTCTCTAGCAGAGAAGAGT-MGB	Alfagene

Table 4 - Reaction mixes and PCR conditions for qPCR to quantify total proviral DNA.

CD3	Gag	PCR conditions
2µL H2O 10µL TaqMan® Gene Expression Master Mix (Applied Biosystems) 2µL CD3 primer mix (F+R, 4µM each) 1µL CD3 probe 5µM 5µL DNA (PCR product diluted 1:50)	2µL H2O 10µL TaqMan® Gene Expression Master Mix (Applied Biosystems) 2µL Gag primer mix (F+R, 4µM each) 1µL Gag probe 5µM 5µL DNA (PCR product diluted 1:50)	95ºC 2min 20 cycles of: 95ºC 30sec 60ºC 30sec 72ºC 2min 72ºC 5min 4ºC hold

3.11 gag mRNA Quantification

In order to quantify gag mRNA in infected TOCs, cDNA was produced from 50ng of RNA purified using ZR-Duet DNA/RNA MiniPrep kit (Zymo research). 50µM of Oligo$(dT)_{20}$ (Life Technologies) and 10mM of dNTP mix (Invitrogen) were added to 11µL of diluted RNA. The mix was incubated for five minutes at 65ºC in T100™ Thermal Cycler (BioRad) and cooled down at 4ºC. Afterwards, 4µL of 5X First Strand RT Buffer (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl2; Invitrogen), 5mM of DDT (Invitrogen), 2U/µL of RNase Out (Invitrogen) and 10U/µL of Superscript III® Reverse Transcriptase (Invitrogen) were added to the mix and incubated for 50 minutes at 50ºC and at 75ºC for 15 minutes. cDNA was cooled down and diluted 1:5. The same primers and probes of Gag (Table 3) were used to quantify the total viral DNA, were used to quantify the gag mRNA in TOCs infected whether with HIV-1 or HIV-2. Standard curves using the plasmids containing the HIV-1 or HIV-2 Gag amplicons or cDNA (for GAPDH) were used for quantification purposes. GAPDH was used as a housekeeping gene for the experiment. The table below shows the PCR reaction mixes, probes and primers used in the process. The quantification was performed by RT-qPCR in ABI 7500 Fast (ThermoFisher Scientific).

Table 5 - Reaction mixes and RT-qPCR conditions to quantify Gag mRNA.

GAPDH	Gag	PCR conditions
4µL H2O 10µL TaqMan® Gene Expression Master Mix (Applied Biosystems) 1µL GAPDH mix (Invitrogen) 5µL cDNA (PCR product diluted 1:5)	2µL H2O 10µL TaqMan® Gene Expression Master Mix (Applied Biosystems) 2µL Gag primer mix (F+R, 4µM each) 1µL Gag probe 5µM 5µL DNA (PCR product diluted 1:5)	95ºC 2min 20 cycles of: 95ºC 30sec 60ºC 30sec 72ºC 2min 72ºC 5min 4ºC hold

3.12 Immunohistochemistry Staining

TOCs were embedded in paraffin after preservation in 4% formaldehyde. Paraffin blocks were cut into sections of 3µm and deparaffinized with xylene and rehydrated with alcohol. Antigen retrieval (pH9) was perfomed by microwave heat at 800 watts for 15 minutes. Sections from TOCs infected with HIV-1 and HIV-2 were stained with anti-p24 (KAL-1, NIH) or anti-p27 (55-2F12, NISCB), respectively, for one hour at room temperature. Next, they were washed with PBS three times for five minutes each. Sections were counterstained with hematoxylin, dehydrated with alcohol and cleared with xylene. In order to assess overall structure a hematoxylin/eosin (H&E) staining was also performed in different sections. Slides were mounted with Quick-D mounting medium (Klinipath) and images (100x, 200x and 400x) were acquired with a Leica DM2500 brightfield microscope.

4 Results

4.1 Virus Production

In order to evaluate the impact of HIV-2 in lymphoid tissue, we first produced a viral stock of HIV-1 and HIV-2 primary isolates with different coreceptor usage. We used primary isolates strains because they are genetically closer to the viruses isolated from HIV-infected individuals, which will allow us to better replicate the infection *in vitro*. Lab-adapted strains are in fact extensively used in studies regarding HIV infection and were once also a primary isolate collected from an HIV-infected patient, however being coculture with uninfected PBMCs and cell lines several times leads to a change on the composition of the original viral population[133].

We produced R5- and X4- tropic HIV-1 and HIV-2 primary isolates by infecting Pha-stimulated uninfected PBMCs and culturing them for 20 days. The viruses were quantified by SG-PERT and Table 6 shows the concentrations obtained for the undiluted and 1:10 diluted samples. Since higher concentrations have been reported to be related to some degree of inhibition of the quantification reaction[130], we decided to use the quantification of the diluted samples to pursuit our investigation.

Table 6 - Concentration of the viruses produced and quantified by SG-PERT.

	Virus	**Quantity mean** (pg/µL)
	$HIV\text{-}1_{92US660}$	2422
	$HIV\text{-}1_{92HT599}$	884
	$HIV\text{-}2_{60415k}$	2491
	$HIV\text{-}2_{20.04}$	2619
Diluted 1:10	$HIV\text{-}1_{92US660}$	10076
Diluted 1:10	$HIV\text{-}1_{92HT599}$	2354
Diluted 1:10	$HIV\text{-}2_{60415k}$	6966
Diluted 1:10	$HIV\text{-}2_{20.04}$	5449

We further confirmed that the viruses produced were infectious using activated PBMCs from healthy donors. Seven days of culture after infection the Gag protein produced was quantified at the single-cell level by flow cytometry using an antibody (KC57) that detects both HIV-1 and HIV-2 Gag[131,132]. The percentage of Gag^+ ($KC57^+$) cells was determined

within the $CD3^+CD8^-$ population, since CD4 is known to be downregulated in $CD4^+$ T cells upon HIV infection[42,134,135] (Figure 9). All viruses were able to replicate in activated $CD4^+$ T cells ($CD3^+CD8^-$) (Figure 9), and could therefore be used in our studies.

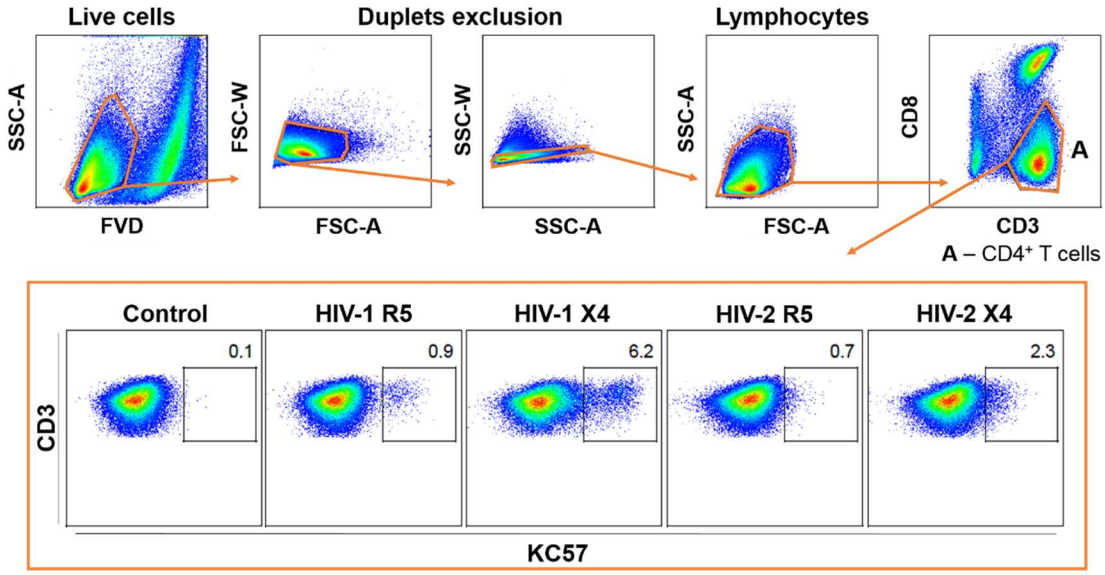

Figure 9 - The viruses produced are able to replicate in activated PBMCs. PBMCs from healthy donors stimulated for three days with Pha were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates and cultured for seven days. Cells were stained with anti-human MAbs and Gag protein quantification was performed at the single-cell level by flow cytometry using KC57 antibody. The percentage of $KC57^+$ cells was determined within $CD3^+CD8^-$ cells. $CD3^+CD8^-$ cells within lymphocytes were gated after exclusion of dead cells by FVD (fixable viability dye) and dublets by side and forward scatter parameters.

4.2 Optimization of TOCs

In order to optimize TOC cultures for HIV infections, TOCs were cultured for 11 days on appropriate membranes in MC and their structure assessed at days 0, 7 and 11 through an hematoxylin/eosin staining. The staining revealed the presence of some germinal centers on day 0 (Figure 10), although they were not always observed as a result of the small pieces of tonsil in culture. On days 7 and 11, the sections showed a loss of cell viability and germinal centers could no longer be found (Figure 10). The external surface of the tonsil is protected by a stratified squamous epithelium[136] and on days 7 and 11 the epithelium was clearly observed on the tonsil sections.

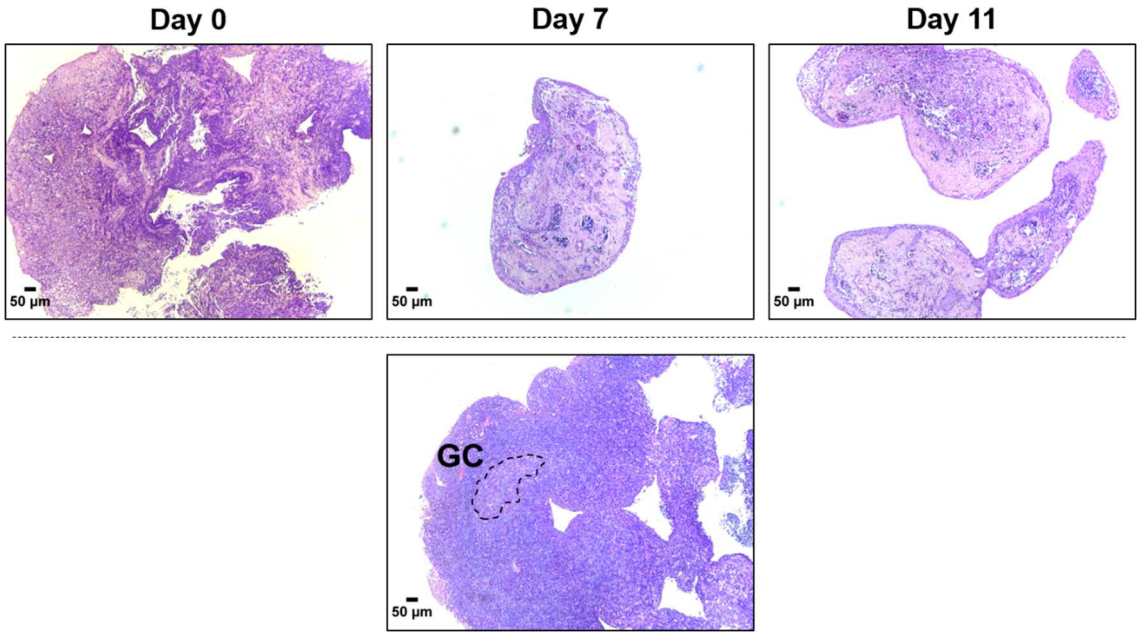

Figure 10 - Structure of TOCs at days 0, 7 and 11. TOCs were cultured in MC during 11 days on Millipore inserts. At day 0, 7 and 11, TOCs were embedded in paraffin and sections were cut and stained with hematoxylin and eosin. The bottom figure is representative of a TOC section containing a germinal center. Data was acquired with a Leica DM2500 brightfield microscope. Amplification: 100x.

We also analyzed cell viability and phenotype at days 0, 2, 4, 7 and 11 by flow cytometry. We applied the same gating strategy to all analysis so that we could compare the populations at all time points (Figure 11a). We observed that cell viability decreased significantly between day 0 and day 2, however it was maintained constant until day 7, decreasing again on day 11 (Figure 11b).

We were also interested in analyzing the cell populations important to study HIV infection in lymphoid tissues.

B cells, which are localized in germinal centers and are important to produce antibodies were diminished between day 0 and day 2, but were preserved until day 11 (Figure 11b), although a large variability was observed between TOCs. The depletion of B cells might be explained by the loss of germinal centers in the tissue during culture which we showed previously, or by the fact that we could not always visualize germinal centers in the small pieces of tonsil.

HIV has a preference to infect $CD4^+$ T cells, mainly the memory subset ($CD45RA^-$), therefore we needed to understand the dynamics of these populations in our cultures. Our data shows that the frequency of $CD4^+$ T cells was maintained, although the memory $CD4^+$ T cell subset decreased over time (Figure 11b).

We also analyzed the frequency of T_{FH} cells in TOCs. During the eleven days of culture, the populations characterized by PD-1 and CXCR5 expression were variable among TOCs (Figure 11c). T_{FH} cells are known to be presented in germinal centers which are lost during culture or not found in TOCs. These results might explain the inconsistencies in this population. Interestingly, we observed a tendency for the PD-1^-CXCR5^+ population to increase between day 0 and day 2, and this is clearly visible in the dotplots (Figure 11c). In contrast, the PD-1^+CXCR5^- decreased over time (Figure 11c).

Therefore, although the tissue was preserved between 7 and 11 days in culture, cell viability was better at day 7. We thus chose this time-point for future HIV infections. Furthermore, we noticed that two TOCs per condition analyzed separately wouldn't allow us to understand what happens in the tissue because they were very heterogeneous among them. For that reason, we performed a new optimization, this time culturing five TOCs during 7 days, in order to have a better representation of the tissue.

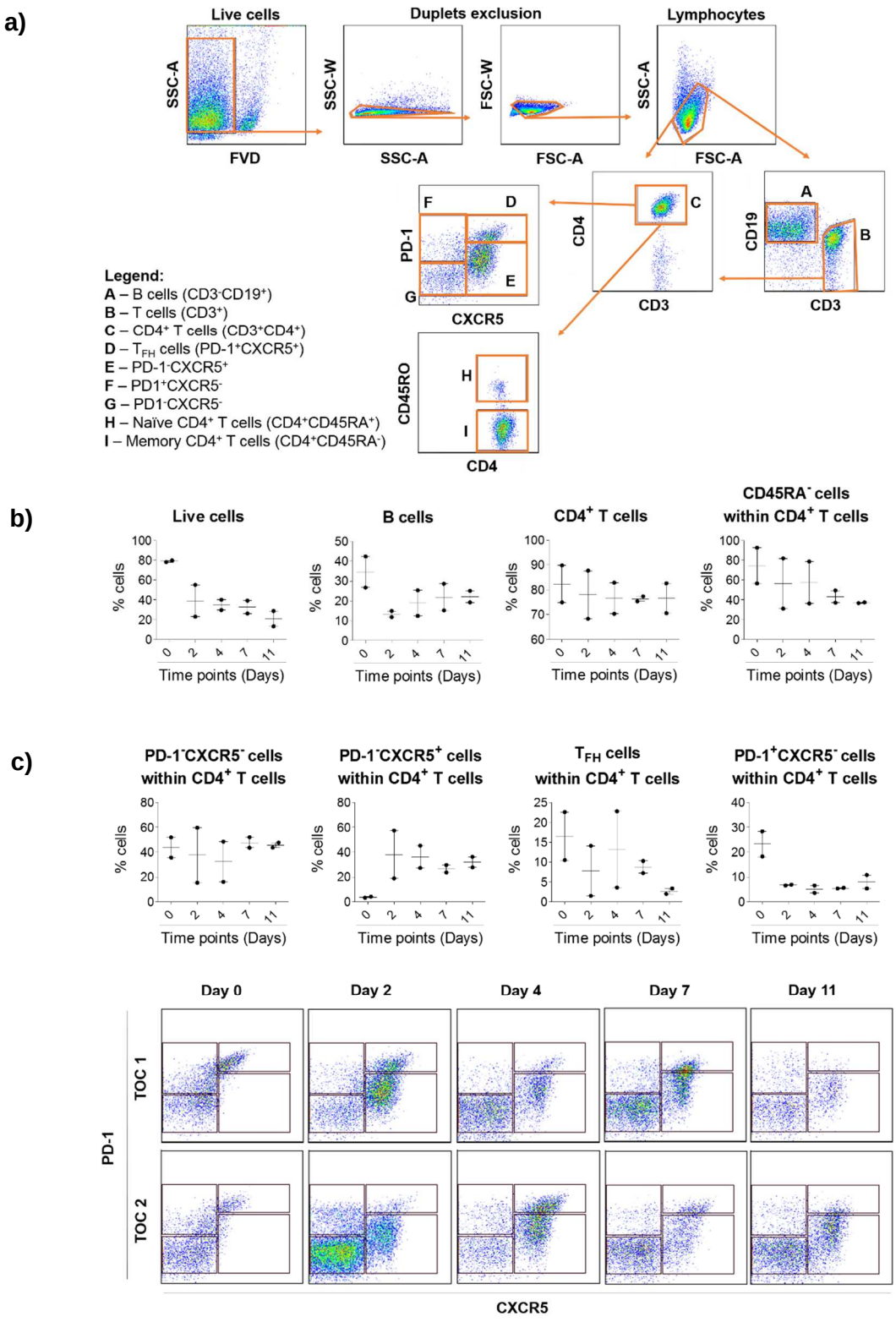

Figure 11 - Cell phenotype in TOCs changes during culture. TOCs were cultured in MC for 11 days on Millipore inserts. At day 0, 2, 4, 7 and 11, two TOCs were collected, mashed and cells were stained with anti-human MAbs and FVD. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar) and GraphPad Prism. Gate strategy to analyze the cell phenotype and viability of the TOCs at days 0, 2, 4, 7 and 11 (a). Live cells, B cells ($CD3^+CD19^-$), $CD4^+$ T cells and memory $CD4^+$ T cells ($CD45RA^-$) were analyzed accordingly with the gating strategy shown above (b). The subsets of PD-1 and CXCR5 were analyzed within $CD4^+$ T cells (c). Each dot represents a single TOC. Lines indicate mean±SEM (standard error of the mean).

We analyzed cell viability and phenotype by flow cytometry and compared five TOCs individually or pooled together at days 0 and 7 (Figure 12). We observed that the population distributions in cells from pooled TOCs were comparable to the mean observed when they were analyzed individually, despite their heterogeneity. We thus concluded that a pool of TOCs would be representative of the tissue and would allow the correct evaluation of the impact of HIV infection.

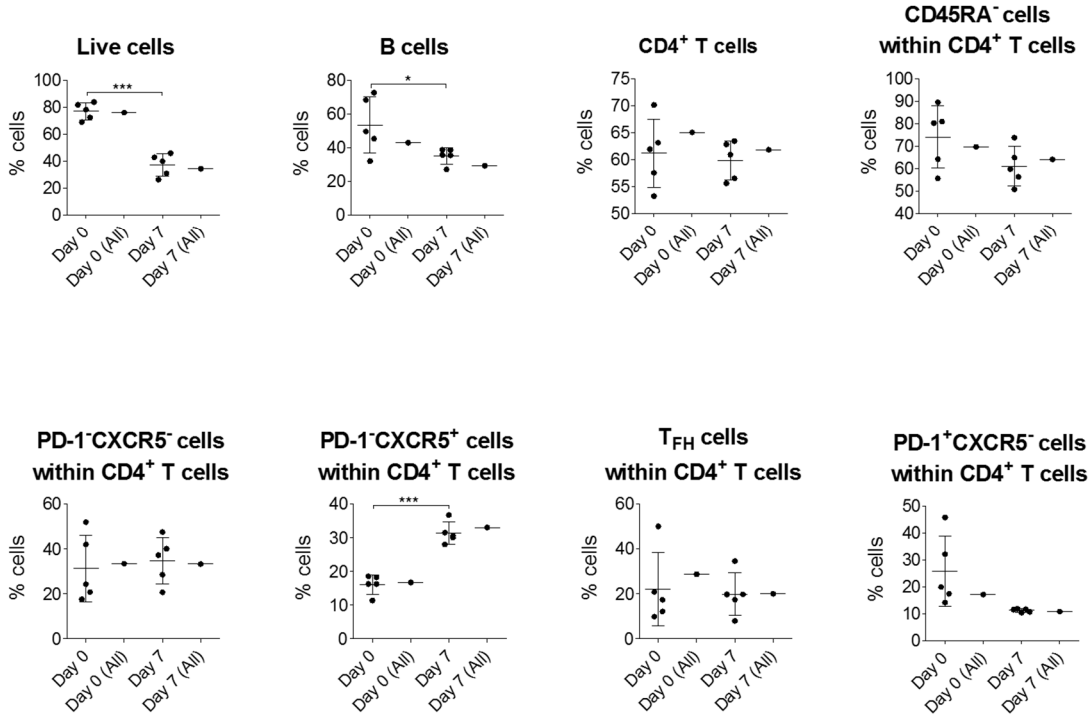

*Figure 12 - The population distributions in cells from pooled TOCs were comparable to the mean observed when they were analyzed individually, despite their heterogeneity. TOCs were culture in MC for seven days in Millipore inserts. At day 0 and day 7, five TOCS were collected, mashed and cells were stained with anti-human MAbs and FVD. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar) and GraphPad Prism. Each dot represents a single TOC. Lines indicate mean±SEM. Statistical analysis was performed by parametric paired T test, *,p<0.05, ***, p<0.001.*

4.3 CXCR4 and CCR5 Expression in $CD4^+$ T cells and T_{FH} Subsets

Given the fact we are using viruses with different coreceptor usage, we questioned the expression of R5 and X4 coreceptors in our populations of interest (Figure 13). We found that T_{FH} cells expressed significantly higher levels of CXCR4 than all the other populations analyzed. In addition, T_{FH} expressed high levels of CCR5, although the highest expression of this coreceptor was found in $PD\text{-}1^+CXCR5^-CD4^+$ T cells. Of note, since the two markers were used in different fluorescence channels, their MFIs cannot be compared.

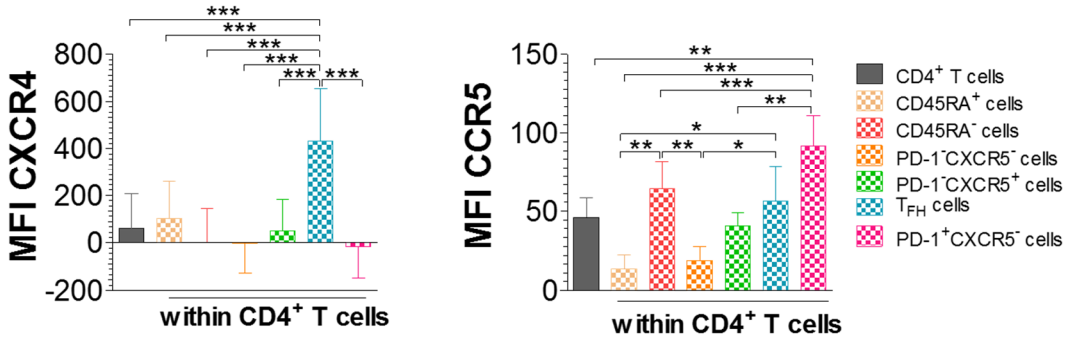

*Figure 13 - TFH and PD-1$^+$CXCR5$^-$ cells have a higher intensity of CXCR4 and CCR5, respectively. A piece of tonsil was mashed and cells were stained with validated antibodies and FVD and acquired at a LSRFortessa cell analyzer (BD Biosciences). CXCR4 and CCR5 were analyzed by flow cytometry within CD4$^+$ T cells, memory (CD45RA$^-$) CD4$^+$ T cells and naïve (CD45RA$^+$) CD4$^+$ T cells as well as in the PD-1/CXCR5 subsets after exclusion of the dead cells by FVD and dublets by side and forward scatter parameters. MFI: median fluorescence intensity. Lines indicate mean±SEM. Statistical analysis was performed by One-way repeated measures ANOVA test with Bonferroni's multiple-comparison posttest, *, p<0.05, ***, p<0.001.*

4.4 HIV-2 Infection of TOCs

In order to investigate the impact of HIV-2 infection in lymphoid tissues TOCs were infected with the R5- or X4-tropic HIV-1 or HIV-2 primary isolates produced. Tissue structure as well as Gag production were assessed by immunohistochemistry on day 7 after culture. Given the quantification of Gag production in infected TOCs would be quite challenging, we could only take qualitative conclusions from the immunohistochemistry results.

We observed that both R5- and X4-tropic HIV-2 primary isolates were able to replicate in the tissue, and that Gag protein expression was both cell-associated and extracellular (Figure 14). Since TOCs were infected with a drop of virus, we questioned whether this extracellular staining represented the virus that remained in the tissue. We thus performed a kinetic analysis of Gag production in TOCs, which confirmed that the extracellular staining found at day 7 was indeed virus produced during culture by HIV-infected cells, since in day 2 we could only observe cell-associated Gag expression (Figure 15).

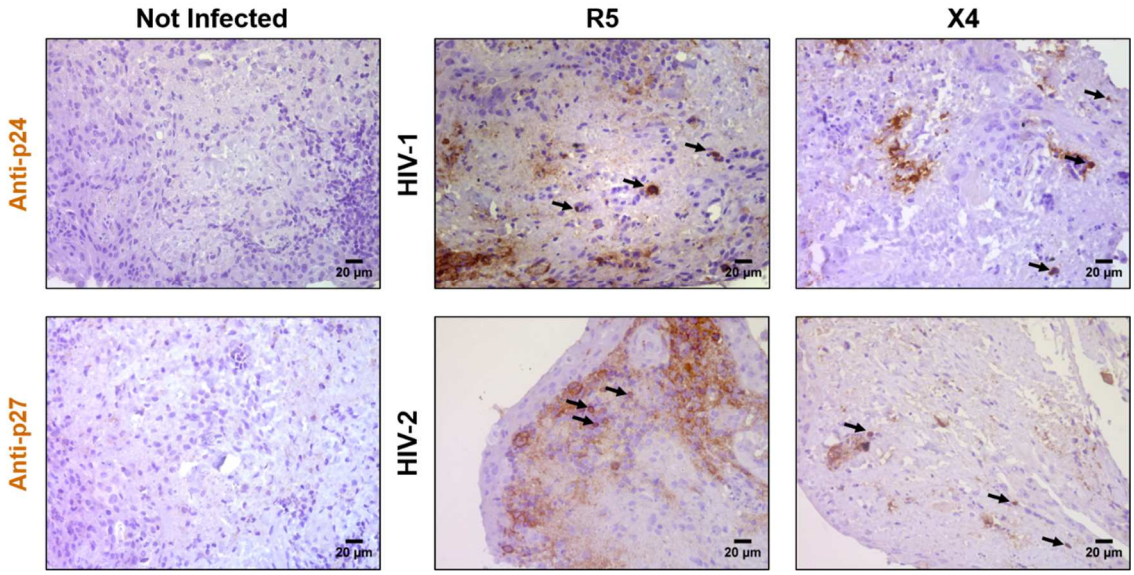

Figure 14 - HIV-2 is able to replicate human tonsillar tissue. TOCs were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates and cultured for seven days on Millipore inserts. Viral production was assessed by immunohistochemistry using anti-Gag antibodies (brown) and hematoxylin counterstain (blue) at day seven. The arrows indicate cell-associated Gag protein expression. Amplification: 400x

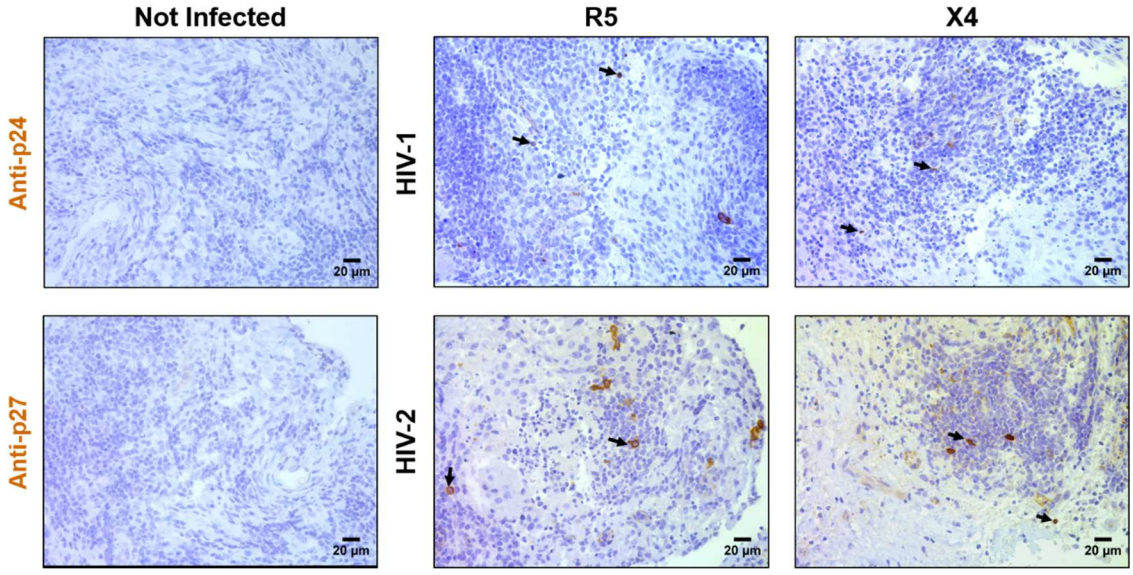

Figure 15 - Gag protein expression is cell associated at day 2. TOCs were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates and cultured for two days. Viral production was assessed by immunohistochemistry using anti-Gag antibodies (brown) and hematoxylin counterstain (blue) at day 2. The arrows indicate cell-associated Gag protein expression. Amplification: 400x

Next we assessed total HIV DNA of cells isolated from HIV-2 or HIV-1-infected TOCs, which are mostly lymphocytes (Figure 11), through quantification of cell-associated viral DNA by RT-qPCR (Figure 16). Our results show that HIV-2 primary isolates with either R5 or X4 coreceptor usage were able to infect the tissue lymphocytes at similar levels, and that those levels were not significantly different from those determined for HIV-1 primary isolates.

Next, we determined the levels of ongoing viral transcription by measuring viral gag mRNA (Figure 16). We found that X4-tropic HIV-1 had significantly higher gag mRNA levels than the other viruses used in the study. In contrast, X4-tropic HIV-2 presented significantly low gag mRNA levels, despite the high levels of total HIV DNA.

We further quantified the viral production at the single-cell level within infected $CD4^+$ T ($CD3^+CD8^-$) cells by flow cytometry (Figure 16). We used the anti-Gag antibody KC57, which was previously shown to detect both HIV-1 and HIV-2 Gag[131,132]. Infection with X4-tropic HIV-1 yielded the highest levels of intracellular Gag protein in isolated cells compared to the other viruses while HIV-2 infection was associated with low levels of Gag production and was coreceptor-independent.

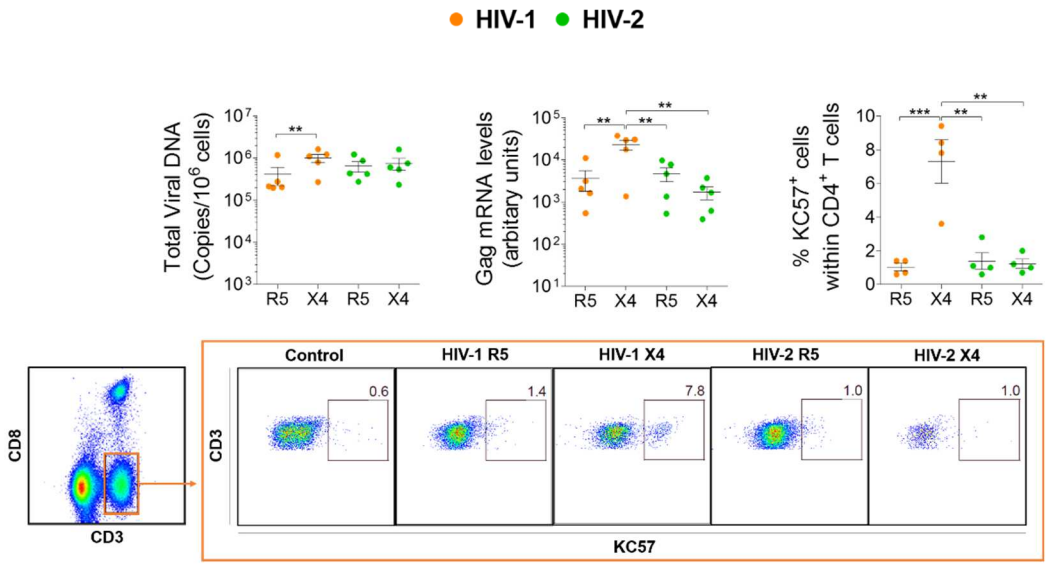

*Figure 16 - HIV-2 infects and replicates in human tonsillar tissue. TOCs were infected with HIV-1 or HIV-2 primary isolates with different coreceptor usage and cultured for seven days. Total viral DNA and gag mRNA levels were determined by qPCR and RT-qPCR, respectively. The level of mRNA expression was normalized to the level of GAPDH expression. Viral production at the single-cell level was assessed by flow cytometry using validated antibodies, including the anti-Gag antibody KC57, within the $CD4^+$ ($CD3^+CD8^-$) population. $CD3^+CD8^-$ cells within lymphocytes were gated after exclusion of dead cells by FVD and dublets by side and forward scatter parameters. Each dot represents a single tonsil. Lines indicate median±SEM. Statistical analysis was performed by One-way repeated measures ANOVA test with Bonferroni's multiple-comparison posttest, **, p<0.01, ***, p<0.001.*

Our data showed that HIV-2 primary isolates were able to infect lymphoid tissue but that the levels of replication in isolated lymphocytes were significantly lower.

4.5 Impact of HIV-2 Infection on TOCs

We also assessed the impact of HIV-2 vs. HIV-1 infection on the lymphocyte cell populations of the human tonsillar tissue in infected TOCs (Figure 16). First, we assessed the cell viability of the cultures after seven days. Interestingly, both X4-tropic HIV-1 and HIV-2 isolates had the lowest percentage of live cells, indicating the higher cytopathicity of the X4 viruses in the lymphoid tissue. We also observed a decrease in

the percentage of $CD4^+$ T cells in all infections compared to the control, and this was particularly striking in infections with X4 viruses.

Next, we analyzed the depletion of naïve vs. memory, defined as: $CD45RO^-$ and $CD45RO^+$, respectively. The results showed that all viruses were able to deplete the memory compartment, but that the depletion was more striking in X4 virus infections.

We next investigated the $CD4^+$ T cell turnover through the expression of the nuclear factor Ki67, which is up-regulated in all cell cycle phases except G_0[67]. All viruses had lower frequencies of proliferating cells, although the X4-tropic viruses showed a tendency for higher proliferation levels.

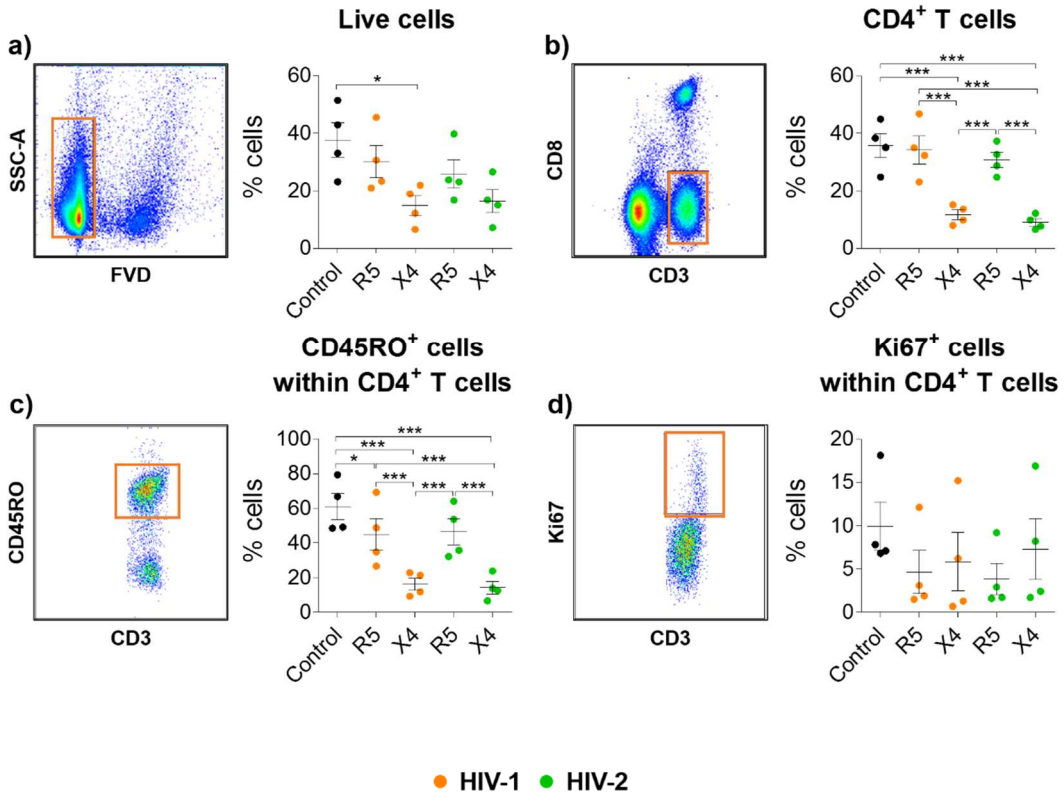

*Figure 17 - The depletion of $CD4^+$ T cells and of the memory subset was coreceptor-dependent. TOCs were infected with HIV-1 or HIV-2 primary isolates with different coreceptor usage and cultured for seven days. Cells were stained with validated antibodies and acquired at a LSRFortessa cell analyzer (BD Biosciences). Dead cells were excluded by FVD (a). The impact of infection was assessed within $CD3^+CD8^-$ T cells due to the downregulation of the recepetor CD4 (b). Memory ($CD45RO^+$) and naïve ($CD45RO^-$) cells were analyzed within $CD3^+CD8^-$ T cells (c). Proliferating cells were assessed by the expression of the marker Ki67 and analyzed with $CD3^+CD8^-$ T cells (d). Data was analyzed with FlowJo Software (TreeStar) and statistical analysis was performed by One-way repeated measures ANOVA test with Bonferroni's multiple-comparison posttest, *, p<0.05, ***, p<0.001. Each dot represents one tonsil. Lines indicate mean±SEM.*

4.6 HIV-2 Infection of T_{FH} Cells

We further assessed the impact of HIV-2 vs. HIV-1 infection on the $CD4^+$ T cell populations defined based on PD-1 and CXCR5 expression with particular focus on T_{FH}

since they represent an important target of HIV infection (Figure 18). We observed that T_{FH} were significantly depleted in all HIV-2 or HIV-1-infected TOCs, but more strikingly with the X4-tropic viruses. On the other hand, PD-1$^-$CXCR5$^-$ cells were preserved. The majority of these cells were naïve CD4$^+$ T cells (%CD45RO$^-$: 79.35%±6.09%), which correlates with the fact that the viruses mostly depleted the memory subset. The expression of CXCR5 seemed to increase during culture as observed in the optimizations. However, only the R5-tropic viruses had a tendency to increase the PD-1$^-$CXCR5$^+$ population. Regarding the population PD-1$^+$CXCR5$^-$, only the X4-tropic viruses increased the percentage of these cells. In the literature, these cells are described as activated T cells, which indicates that these viruses might have more.

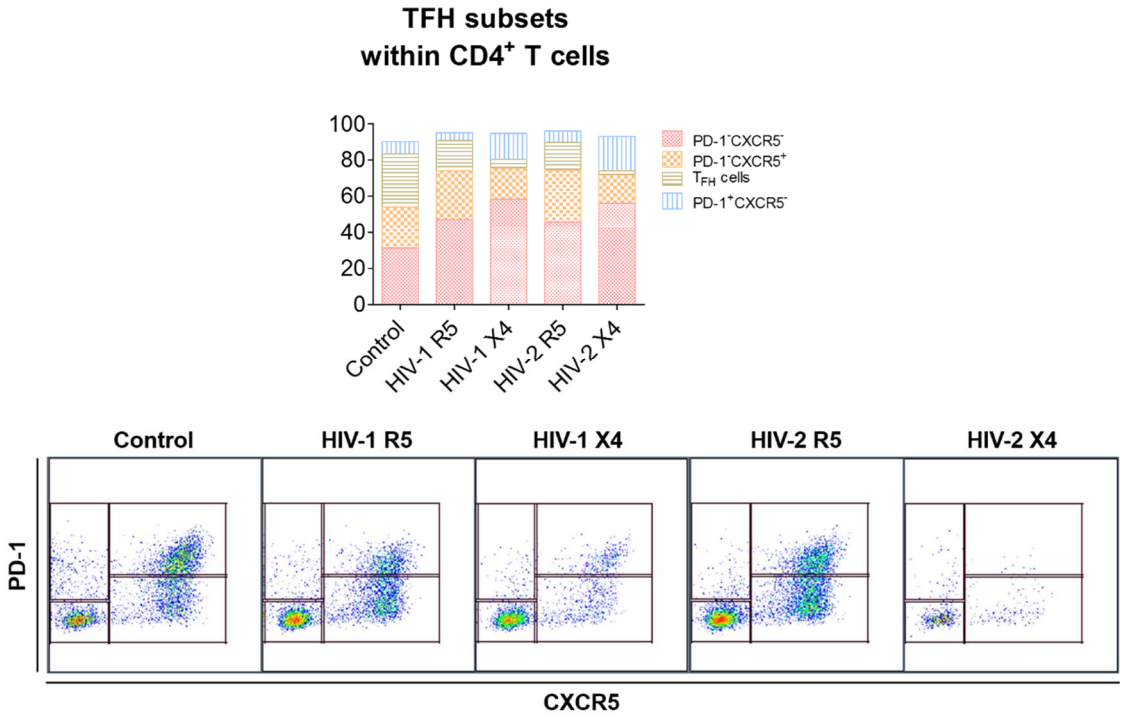

*Figure 18 - TOCs infected with X4-tropic viruses lose T_{FH} cells due to their higher expression of CXCR4 coreceptor. TOCs were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates and cultured for seven days. TOCs were mashed, cells were stained with validated antibodies and acquired on a LSRFortessa cell analyzer (BD Biosciences). Data was analyzed with FlowJo Software (TreeStar). The percentage of T_{FH} subsets was analyzed with GrapPad Prism. Statistical analysis was performed by One-way repeated measures ANOVA test with Bonferroni's multiple-comparison posttest, *, p<0.05, **, p<0.01, ***, p<0.001. Each dot represents a single tonsil. Lines indicate mean±SEM.*

4.7 HIV-2 Infection of FoxP3$^+$ Cells

Finally, we studied the impact of HIV-2 vs. HIV-1 on tonsillar Foxp3$^+$ cells. We found that X4-tropic viruses induced higher levels of Foxp3 expression, particularly HIV-2 (Figure 19a). The higher frequency of Foxp3$^+$ cells was not due to higher proliferation of these populations, since Ki67 expression was not increased in HIV-infected cells (Figure 19b).

The existence of a subset of follicular cells that expresses the Foxp3 transcription factor, the T_{FR} cells, has been recently reported. Since $Foxp3^+$ cells were higher in the X4-tropic viruses, we investigated whether those cells were follicular ($CXCR5^+$) $Foxp3^+$ cells (Figure 19c). X4-tropic HIV-2 induced significantly higher depletion of follicular cells within $Foxp3^+$ cells, indicating that most of the $Foxp3^+$ cells that persisted in infected TOCS were Tregs and not T_{FR}.

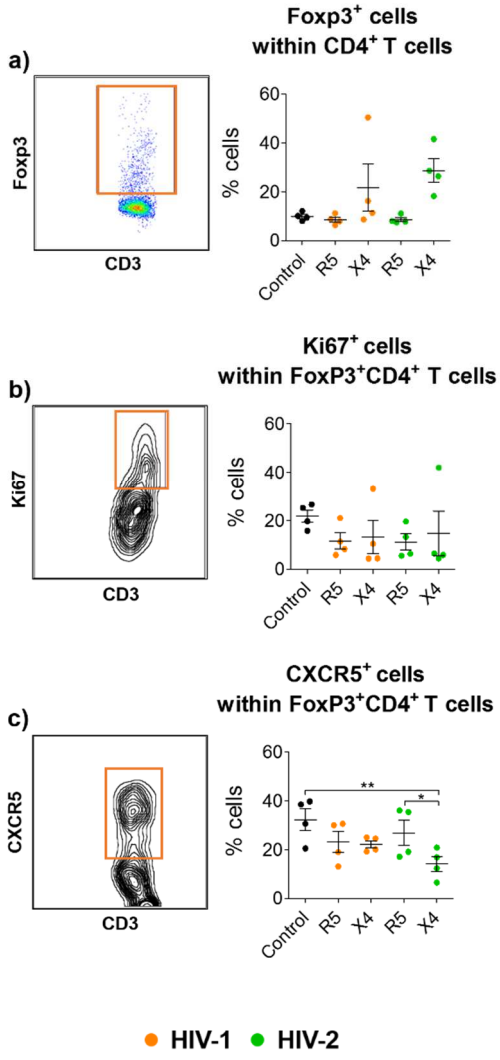

*Figure 19 - HIV-infected TOCs showed a depletion of follicular cells ($CXCR5^+$) within $Foxp3^+$ cells which is more striking in TOCs infected with HIV-2 X4. TOCs were infected with R5- or X4-tropic HIV-1 or HIV-2 primary isolates and cultured for seven days. TOCs were mashed, cells were stained with validated antibodies and acquired on a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed with FlowJo Software (TreeStar). $Foxp3^+$ cells were analyzed within $CD4^+$ T cells (a). $Ki67^+$ cells (b) and $CXCR5^+$ cells (c) were analyzed within $Foxp3^+$ cells. Statistical analysis was performed by One-way repeated measures ANOVA test with Bonferroni's multiple-comparison posttest, **, $p<0.01$. Each dot represents a single tonsil. Lines indicate mean±SEM.*

5 Discussion and Future Perspectives

This project determined the impact of HIV-2 on human tonsillar tissue. Our data suggested that both HIV-2 and HIV-1 were able to infect and replicate in lymphoid tissue and that cytopathicity was coreceptor dependent. However, HIV-2 primary isolates showed a lower level of replication on lymphocytes as compared to HIV-1.

Immunohistochemistry in infected tissue showed that both R5- and X4-tropic HIV-1 and HIV-2 primary isolates were able to infect and replicate in human tonsillar tissue. This was supported by the high levels of total viral DNA found in lymphocytes isolated after tissue infection, which were consistent with previous studies where high levels of HIV DNA were described in LMNCs (lymph node mononuclear cells) from both HIV-1 and HIV-2-patients[20].

Interestingly, when we evaluated the levels of gag mRNA and Gag protein at the single-cell level, we found that only X4-tropic HIV-1 primary isolate produced significant levels of viral mRNA and protein. Thus, even though, HIV-2 was able to infect the tissue at the same extent as compared to X4-tropic HIV-1, viral replication in lymphocytes was lower.

We also determined the level of cytopathicity of the viruses in the human tonsillar tissue, by analyzing the level of cell depletion. We showed that X4-tropic viruses were more cytopathic than the R5-tropic HIV-1 and HIV-2. Penn, M. L. *et al.* (1999) had already shown that X4 HIV-1 massively depleted $CD4^+$ T cells whereas R5 viruses depleted such cells only mildly, despite comparable viral replication. Afterwards, Schramm *et al.* (2000) determined the cytopathicity of HIV-2 infection in TOCs and showed the cytopathicity was comparable to HIV-1 and coreceptor dependent, in agreement with our data.

Furthermore, we analyzed other important cell subsets which are known to be affected by HIV infection. The memory subset of $CD4^+$ T cells is known to be permissive to HIV-1 and HIV-2 infection[29,31,32]. Indeed, we found that all viruses were able to deplete memory $CD4^+$ T cells, and that this depletion was coreceptor-dependent as it was more severe in infections with X4-tropic viruses. On the other hand, Dai, J. *et al.* (2009) showed that the level of integration of HIV-1 in naïve cells was lower than in memory $CD4^+$ T cells and Soares, R. *et al.* (2006) showed the same in HIV-2 infected individuals which explains the depletion of $CD45RO^+CD4^+$ T cells by the viruses.

In our study, there was a trend for the decrease of proliferating cells in lymphocytes isolated from TOCs infected with either HIV-1 or HIV-2. This is in contrast to what has been described in the blood of HIV-infected patients. HIV-1 and HIV-2 infected patients

with the same levels of $CD4^+$ T cell depletion, but large differences in viremia exhibited similar elevations in the frequencies of activated and cycling T cells[13,34] Hazenberg, M. D. *et al.* (2000) also showed that in untreated HIV-1 infection, the percentage and number of circulating $Ki67^+CD4^+$ lymphocytes was significantly increased, compared with values obtained from healthy individuals. Our data showed that X4-tropic viruses had a similar total HIV DNA and a higher depletion of $CD4^+$ T cells compared to R5-tropic viruses. We would expect more immune activation, therefore a higher level of proliferating cells in both X4-tropic viruses. However, we are studying a tissue outside its environment where there is a limited number of cells, while in the human body the cells are circulating and proliferating. Moreover, we showed that the viruses depleted significantly the memory $CD4^+$ T cells which are usually proliferating.

Our data regarding the impact of HIV-2 vs. HIV-1 infection of tonsillar Treg showed that TOC infection with X4-tropic viruses resulted in higher frequencies of $Foxp3^+$ cells within $CD4^+$ T cells at the end of the culture, while R5 viruses did not alter the Treg frequency, as compared to the non-infected control. We further showed that this was not due to increased Treg proliferation in X4-tropic virus infections. Previously, it was reported that lymphopenic HIV-2 and HIV-1 infected individuals showed a high Treg frequency in PBMCs[35] and in lymphoid tissues[36], which is in agreement with our data.

Here we assessed the impact of infection on $CXCR5^+$ follicular Tregs. Our data showed that there was a depletion of the $CXCR5^+$ population within Foxp3+ cells, which was more striking in X4-tropic HIV-2 infection. The role of T_{FR} is poorly understood in the context of HIV infection. To our knowledge, only Miles, B. *et al.* (2015) focus on T_{FR} in HIV infection and They reported that during infection those cells were expanded through mechanisms involving viral entry.

One of the main reasons why we were interested in studying HIV infection in LOs was due to the role of T_{FH} in this context. Since B cells only are able to survive, expand and undergo differentiation if they encounter a T_{FH} cell[38], T_{FH} cells have a major role in antibody production. T_{FH} have been highly studied in HIV-1 infection[39–42] however, to our knowledge there is no study regarding the impact of HIV-2 infection on GCs. Our data showed that T_{FH} cells ($PD\text{-}1^+CXCR5^+$) were significantly depleted by all viruses, and this was particularly striking with the X4-tropic viruses. Previous studies showed that GC T_{FH} cells are highly permissive to HIV-1[39,40,42], which is in agreement with our data. Moreover, Kohler, S. L. *et al.* (2016) also compared HIV-1 viruses with different coreceptor usage, and determined that both R5- and X4-tropic viruses were highly permissive to HIV-1, but only X4-tropic HIV-1 significantly depleted GC T_{FH}. We further show that T_{FH} cells

express high levels of CXCR4, in agreement with previous reports[42,139], in support of a coreceptor dependency of their infection. Some HIV-2 individuals are able to produce neutralizing antibodies[140], however the X4-tropic HIV-2 we studied depleted significantly T_{FH} cells which may difficult the surviving of B cells. On the other hand, there are some studies that reported in HIV-1 untreated infected individuals an expansion of T_{FH} cell in lymph nodes which they attribute to high immune activation[40,114]. Kohler, S. L. et al. (2016) used *in situ* hybridization for HIV-1 RNA in conjunction with immunostaining for CD20 (a marker for B cells) and IgD (a marker for unswitched B cells) in lymph nodes of untreated individuals, and found that the GCs were decreased in the percentage of total tissue section, however they harbored the most of HIV-1 RNA. These results are controversially, so there are still many questions to be answered regarding the role of T_{FH} cells in the context of HIV infection.

Regarding the PD-1$^-$CXCR5$^+$ population, we observed a decrease in the X4-tropic viruses, compared to the control. The expression of CXCR5 allows these cells to migrate away of the T cell zone towards the B cell follicle [23,24,43], without B cells this subset disappears[26,44]. This might explain the decrease in the PD-1$^-$CXCR5$^+$ population observed in the infections with X4-tropic viruses. Kohler, S. L. *et al.* (2016) suggested during HIV-1 infection both PD-1 and, to a lesser extent, CXCR5 may be downregulated, which might explain the previous results. On the other hand, we observed an increase in the frequency of the PD-1$^-$CXCR5$^-$CD4$^+$ T cells on infected TOCs compared to the control, which was most probably related to the preservation of the naïve population upon HIV-2 or HIV-1 infection in TOCs.

Lymphoid organs are the major reservoir of HIV-1 and HIV-2 infection and are also important to generate immune responses against pathogenes. Therefore, they provide an important model to the study the virus-host relationship. This project allowed us to understand the impact of both infections in the cell populations of the tissue and gave us new insights about HIV immunopathogenesis.

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